

UNIVERSITÉ DU QUÉBEC À MONTRÉAL

UNIVERSITÉ DE LA MÉDITERRANÉE

MOLÉCULES NATURELLES CIBLANT LES INTÉGRINES
ET SPHINGOSINE-1-PHOSPHATE : DE NOUVEAUX RÉGULATEURS DE
L'ANGIOGÈNE CÉRÉBRALE ET DU DÉVELOPPEMENT TUMORAL.

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PAR
ANTHONY PILORGET

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LISTE DES ABBRÉVIATIONS

AAA	agents antiangiogéniques
ADN	acide désoxyribonucléique
ADP	adénosine diphosphate
APC	<i>adenomatous polyposis coli</i>
ARN	acide Ribonucléique
ATP	adénosine triphosphate
BHE	barrière hématoencéphalique
CAM	<i>cell adhesion molecules</i>
CLPs	<i>C-type lectin like proteins</i>
DAOY	cellules de médulloblastome humain
EC	(-) <i>epicatechin</i>
ECG	(-) <i>epicatechin-3-gallate</i>
EDG	<i>endothelial differentiation gene</i>
EGC	(-) <i>epigallocatechin</i>
EGCG	(-) <i>epigallocatechin-3-gallate</i>
FGF	<i>fibroblast growth factor</i>
HBMEC	<i>human brain microvascular endothelial cells</i>
KGD	lysine-glycine-aspartate
KQAGD	lysine-glutamine-alanine-glycine-aspartate
LPA	<i>lysophosphatidic acid</i>
LPP	lysophospholipide phosphatase
LVD	lysine-valine-aspartate
MAPK	<i>mitogen-activated protein kinases</i>
MDR	<i>multidrug resistance</i>
MEC	matrice extracellulaire
MMPs	<i>matrix metalloproteinases</i>
MT1-MMP	<i>membrane type 1-matrix metalloproteinase</i>

MT-MMPs	<i>membrane type-matrix metalloproteinases</i>
NFκB	<i>nuclear factor kappa B</i>
OMS	Organisation Mondiale de la Santé
P-gp	<i>P-glycoprotein</i>
PI3K	phosphatidylinositol-3-kinase
PKA	protéine kinase A
PKC	protéine kinase C
RBE4	cellule endothéliale cérébrale de rat
RGD	arginine-glycine-aspartate
RTD	arginine-thréonine-aspartate
RT-PCR	<i>reverse transcription-polymerase chain reaction</i>
S1P	sphingosine-1-phosphate
SNC	système nerveux central
SphK	sphingosine kinase
TIMPs	tissue inhibitor of metalloproteinases
UV	ultraviolet
VEGF	<i>vascular endothelial growth factor</i>

RÉSUMÉ

Au Canada, 10 000 nouveaux cas de tumeurs cérébrales sont diagnostiqués chaque année. Ces tumeurs représentent la seconde cause de décès dûs au cancer chez les enfants de moins de 15 ans. Par conséquent, la découverte de nouvelles armes thérapeutiques contre ces tumeurs est plus que jamais indispensable afin de combattre plus efficacement ce type de cancer.

La recherche présentée dans cette thèse s'articule autour de deux axes de recherche principaux. Le premier concerne la caractérisation des propriétés antitumorales et antiangiogéniques de deux molécules d'origine naturelle, l'(-) épigallocatechine-3-gallate (EGCG), un polyphénol extrait du thé vert et la lébectine, une lectine de type C, isolée du venin de la vipère *Macrovipera lebetina*. Nous avons volontairement restreint nos travaux à l'étude des effets de ces deux molécules sur l'expression et la fonction des intégrines, protéines d'adhérence cellulaire jouant un rôle majeur dans le développement des tumeurs. Le deuxième axe de recherche de cette thèse s'est focalisé sur l'étude des effets de la sphingosine-1-phosphate (S1P) au niveau de l'endothélium vasculaire cérébral. Nous avons étudié l'impact de la S1P sur le potentiel angiogénique des cellules endothéliales cérébrales et nous avons mis en évidence le rôle de la S1P dans la régulation de la fonction de transport de la P-gp au niveau de l'endothélium cérébral.

Dans un premier temps, nous avons étudié l'effet de l'EGCG sur les propriétés d'adhérence et de migration des cellules DAOY de médulloblastome. Dans cette étude, nous avons démontré que l'EGCG augmente fortement l'adhérence des cellules DAOY sur le collagène de type I, tout en réduisant le potentiel migratoire de ces cellules sur cette même matrice. Nous avons aussi démontré que l'EGCG stimule spécifiquement l'expression génique et protéique de l'intégrine $\beta 1$ dans les cellules DAOY. Ces résultats prouvent que les intégrines représentent une cible moléculaire de l'EGCG. De plus, cette étude permet de penser que l'EGCG pourrait être efficace dans le traitement des médulloblastomes.

Notre seconde étude a permis de caractériser les propriétés antiangiogéniques de la lébectine au niveau des cellules endothéliales cérébrales humaines HBMEC. Nos résultats démontrent que la lébectine inhibe très efficacement l'adhérence et la migration des cellules HBMEC sur la fibronectine et la vitronectine. La lébectine bloque aussi la tubulogénèse des cellules HBMEC sur Matrigel™, et ce, à des concentrations de l'ordre du nanomolaire. Du point de vue moléculaire, nous avons montré que la lébectine interagit spécifiquement avec les intégrines αV et $\alpha 5 \beta 1$ à la surface des cellules HBMEC. Cette interaction bloque les fonctions d'adhérence de ces intégrines, ce qui explique les effets antiangiogéniques de la lébectine. Enfin, en utilisant les tests d'angiogénèse *in vivo* sur membrane chorioallantoïdienne de poulet et en implant sous-cutané de

Matrigel™ chez la souris, nous avons montré que la lébectine inhibe fortement l'angiogenèse *in vivo*. Ces résultats suggèrent que la lébectine pourrait être utilisée très efficacement dans les thérapies antiangiogéniques ciblant les tumeurs cérébrales.

Dans les conditions physiologiques, l'endothélium vasculaire cérébral est au contact de nombreux facteurs de croissance présents dans le sang ou le cerveau. Lors des phénomènes d'hémostase, la concentration plasmatique de sphingosine-1-phosphate (S1P) augmente fortement. Par conséquent, nous nous sommes intéressés aux effets de la S1P sur l'endothélium cérébral. En utilisant les cellules endothéliales cérébrales de rat RBE4, nous avons démontré que la S1P, bien qu'étant un facteur proangiogénique caractérisé, inhibe spécifiquement les propriétés angiogéniques des cellules endothéliales cérébrales. Du point de vue moléculaire, nous avons démontré que l'effet antiangiogénique de la S1P au niveau des cellules RBE4 est relié à l'absence de MT1-MMP, une métalloprotéase de matrice de type membranaire. Cette étude suggère donc que la S1P participe à la protection du cerveau via la stabilisation de l'endothélium cérébral.

Les cellules endothéliales cérébrales participent à la mise en place de la barrière hématoencéphalique (BHE). Celle-ci permet de filtrer efficacement les molécules qui passent du sang vers le cerveau. La BHE joue ainsi un rôle crucial dans la protection du système nerveux central. Afin d'expulser les xénobiotiques hors du cerveau, les cellules endothéliales cérébrales expriment des transporteurs spécifiques, tels que la glycoprotéine P ou P-gp. Dans notre dernière étude, nous avons démontré que la sphingosine kinase, l'enzyme qui permet la synthèse de S1P, régule l'expression et la fonction de transport de la P-gp. De plus, nous avons démontré que la S1P elle-même module l'activité de transport de la P-gp. Ces résultats renforcent l'hypothèse selon laquelle la S1P participe à la protection du cerveau.

L'ensemble de nos résultats a permis d'identifier l'endothélium cérébral comme un tissu cible majeur pour les thérapies antitumorales. De plus, nous avons identifié les intégrines, la sphingosine kinase et la S1P comme des cibles moléculaires importantes pour les traitements antitumoraux et antiangiogéniques. Nos travaux ouvrent donc de nouvelles perspectives pour le développement de nouveaux traitements contre les tumeurs cérébrales.

Mots clés : Angiogenèse tumorale, tumeurs cérébrales, médulloblastomes, intégrines, sphingosine-1-phosphate.

CHAPITRE I

REVUE BIBLIOGRAPHIQUE

1.1. LE CANCER

Les cellules normales fonctionnent entre elles de manière synchrone et harmonieuse. Elles se rassemblent pour former des tissus bien différenciés qui formeront eux-mêmes les organes du corps. Un cancer apparaît lorsqu'une partie des cellules normales commence à se transformer et à se diviser de façon anarchique pour devenir maligne. La transformation cellulaire est un processus long, qui peut durer de 20 à 30 ans. Au cours de cette période, les cellules subissent plusieurs mutations qui mènent au dérèglement cellulaire. Lorsque les cancers sont diagnostiqués, les cellules sont déjà dans une phase de croissance rapide (Figure 1.1). Si le système immunitaire n'est pas capable de détruire ces cellules anormales, leur nombre continue d'augmenter pour former une tumeur tout d'abord bien délimitée. Avec le temps, cependant, la tumeur va envahir les tissus voisins pour les détruire progressivement. Les cellules cancéreuses peuvent également utiliser la voie lymphatique ou sanguine pour atteindre des régions du corps très éloignées de leur point de départ, où elles formeront de nouvelles tumeurs appelées métastases (Figure 1.2). Le mot "cancer" est un terme générique qui désigne près de 150 types de tumeurs différentes affectant les divers organes du corps, le tissu myéloïde (ou moelle osseuse) qui fabrique les éléments figurés du sang ainsi que le système lymphatique.

Au Canada, on estime à 153 100 le nombre de nouveaux cas de cancer et à 70 400 le nombre de décès attribuables au cancer en 2006 selon les données fournies par la Société Canadienne du Cancer (Statistique Canada). En 2006, 989 800 années potentielles de vie se seront volatilisées à cause du cancer, soit près de 32 % de toutes les années potentielles de vie perdues aux mains de toutes les causes de mortalité.

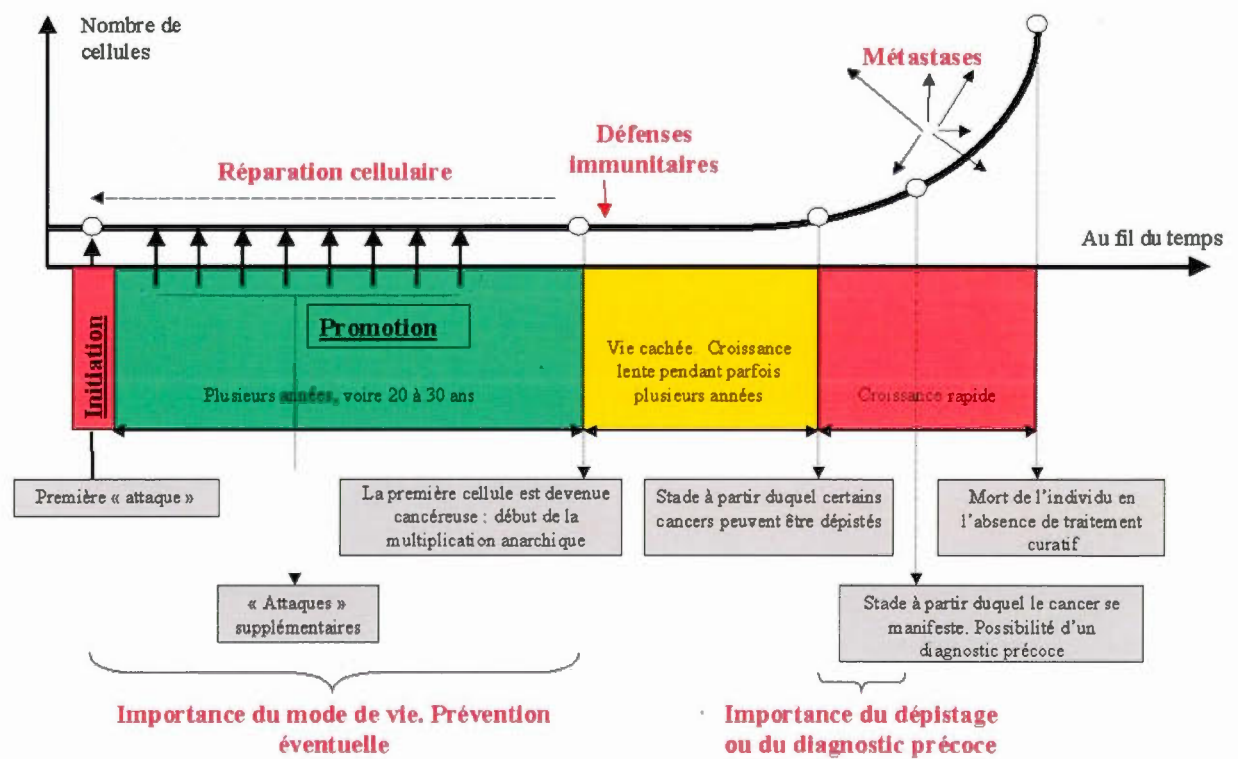


Figure 1.1 : L'évolution chronologique d'un cancer.



1. Prolifération de la tumeur primaire et formation de vaisseaux sanguins pourvoyeurs de nutriments et d'oxygène.
2. Invasion du tissu de soutien et arrivée dans le système sanguin.
3. Les cellules tumorales fragiles s'entourent de plaquettes qui les protègent lors de leur transport.
4. Arrêt dans un organe (par exemple, le foie), adhérence à la paroi vasculaire interne et sortie du vaisseau.
5. Adaptation au nouvel environnement, prolifération et angiogenèse : installation de la métastase.

Figure 1.2 : Les différentes étapes de l'évolution d'un cancer

1.2. LES TUMEURS CÉRÉBRALES

1.2.1. Généralités

Au Canada, 10 000 nouveaux cas de tumeurs cérébrales sont diagnostiqués chaque année. Près d'un tiers de ces cancers sont des tumeurs primaires. Elles constituent une des causes de décès dû au cancer dont la croissance est la plus rapide chez les personnes âgées. Les tumeurs cérébrales représentent la troisième plus importante cause de décès dû au cancer chez les hommes, et la quatrième plus importante cause de décès dû au cancer chez les femmes de 15 à 34 ans. Enfin, elles sont la quatrième plus importante cause de décès chez les hommes de 35 à 54 ans. Toutes ces données épidémiologiques proviennent de la Fondation canadienne des tumeurs cérébrales. Même si les tumeurs cérébrales secondaires ou métastatiques peuvent apparaître à tous les âges, elles se retrouvent le plus souvent dans la population adulte (Gavrilovic and Posner 2005). De plus, les tumeurs cérébrales représentent la seconde cause de mortalité due au cancer chez les enfants. Puisque le cerveau exerce le contrôle de nos sens, de nos sensations et de notre mémoire, les tumeurs qui le touchent représentent une menace importante pour les personnes qui en sont atteintes.

Les tumeurs cérébrales peuvent se développer dans toutes les régions du cerveau et de la moelle épinière. Le cervelet peut aussi être un site tumoral (Figure 1.3). Les principales tumeurs cérébrales sont les astrocytomes, principalement localisés dans le cerveau, les médulloblastomes, touchant presque exclusivement le cervelet et les oligodendrogliomes, localisés eux aussi au niveau du cerveau. Divers adénocarcinomes peuvent aussi se développer au niveau de l'épiphyse et de l'hypophyse, entraînant une altération importante de la production hormonale de ces deux glandes (Figure 1.4).

Les diverses tumeurs du cerveau sont cataloguées selon le type de cellules et de tumeurs. Les gliomes représentent la vaste majorité des tumeurs cérébrales (Ohgaki and Kleihues 2005). Les tumeurs cérébrales sont classées par l'Organisation Mondiale de la Santé (OMS) sur la base de leurs caractéristiques histologiques (Kleihues et al. 1993). Cette classification ainsi que la localisation de la tumeur permettent de préciser le type de tumeur et sa gravité (Figures 1.3 et 1.4). Par exemple, les astrocytomes sont subdivisés en quatre catégories (ou grades) en fonction de leur malignité (Tableau 1.1). Des caractéristiques phénotypiques particulières permettent d'établir le grade des tumeurs. Seuls les grades II, III et IV sont malins. Outre les gliomes, on compte aussi parmi les tumeurs rares du cerveau les médulloblastomes et les épendymomes (McKinney 2005), qui touchent surtout les enfants, de même que les oligodendrogliomes (Baehring 2005) (Kleihues et al. 1993).

Désignation de l'OMS	Grade de l'OMS*	Grade de Kernohan*	Grade de St. Anne/Mayo	Critères de St. Anne/Mayo
Astrocytome pilocytique	I	I	Exclus	-
Astrocytome	II	I, II	1	Aucun critère phénotypique
			2	Atypies nucléaires
Astrocytome anaplasique	III	II, III	3	Atypies nucléaires et une fréquence de division cellulaire élevée.
Glioblastome	IV	III, IV	4	Atypies nucléaires, une fréquence de division cellulaire élevée, une prolifération endothéliale majeure et/ou des zones de nécrose

Tableau 1.1 : Différentes échelles pour définir les grades des astrocytomes

* L'échelle de l'OMS et l'échelle Kernohan ne tiennent pas compte de critères phénotypiques. Les grades mis en place par l'OMS dépendent uniquement de l'observation microscopique des cellules.

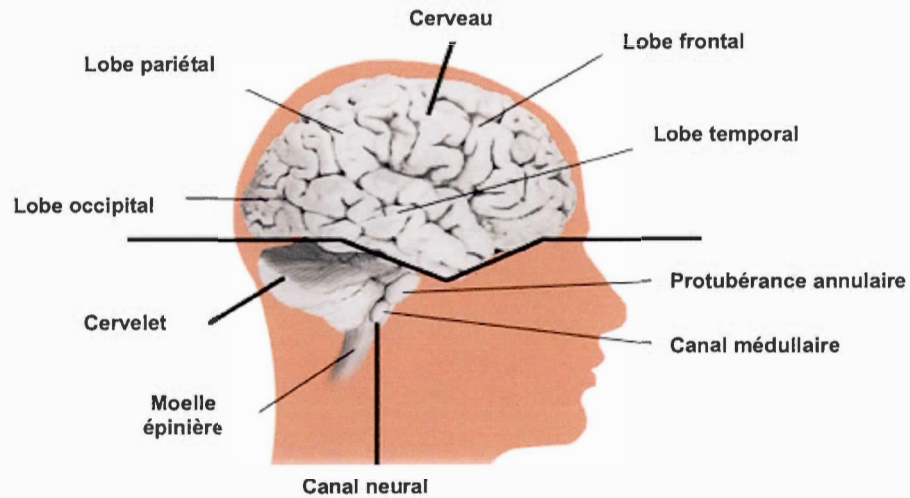


Figure 1.3 : Anatomie du cerveau.

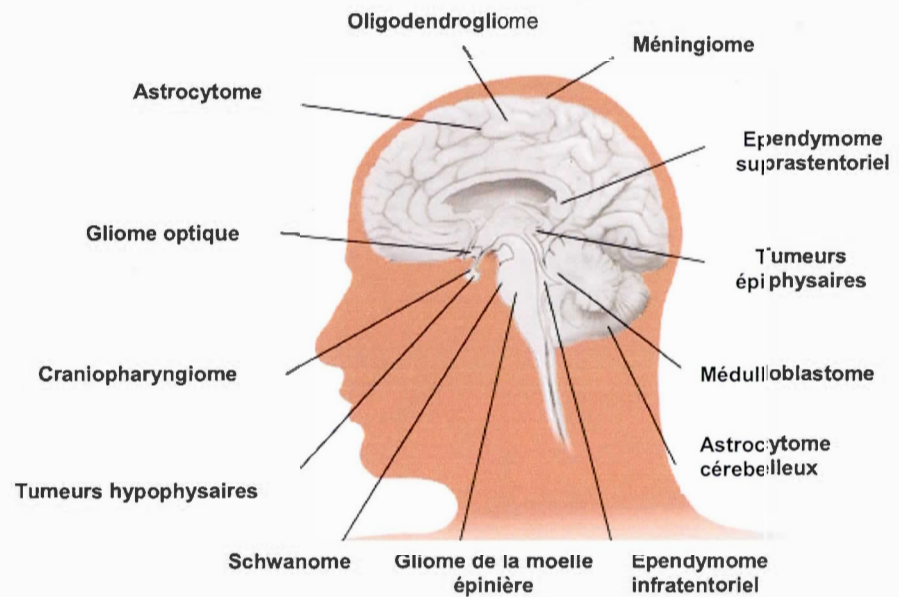


Figure 1.4 : Les principales tumeurs cérébrales.

1.2.2. Les tumeurs cérébrales chez l'enfant.

Les tumeurs cérébrales constituent les plus fréquentes des tumeurs solides chez l'enfant (25% de l'ensemble des tumeurs) (Baldwin and Preston-Martin 2004). Leur incidence est de l'ordre de 2,5 cas pour 100 000 enfants de moins de 15 ans. L'âge du diagnostic se situe avec un maximum de fréquence entre 4 et 8 ans (données de la Fondation canadienne des tumeurs cérébrales). A l'inverse de l'adulte, les métastases sont pratiquement inexistantes à cet âge de la vie. Elles posent des problèmes spécifiques, à la fois d'ordre diagnostique et thérapeutique, sachant la vulnérabilité du tissu cérébral chez l'enfant, et la nécessité de préserver au maximum l'avenir neurologique de ces patients. Selon l'histologie (Figure 1.5), on distingue les médulloblastomes, les astrocytomes, les épendymomes et les craniopharyngiomes (Kleihues et al. 1993).

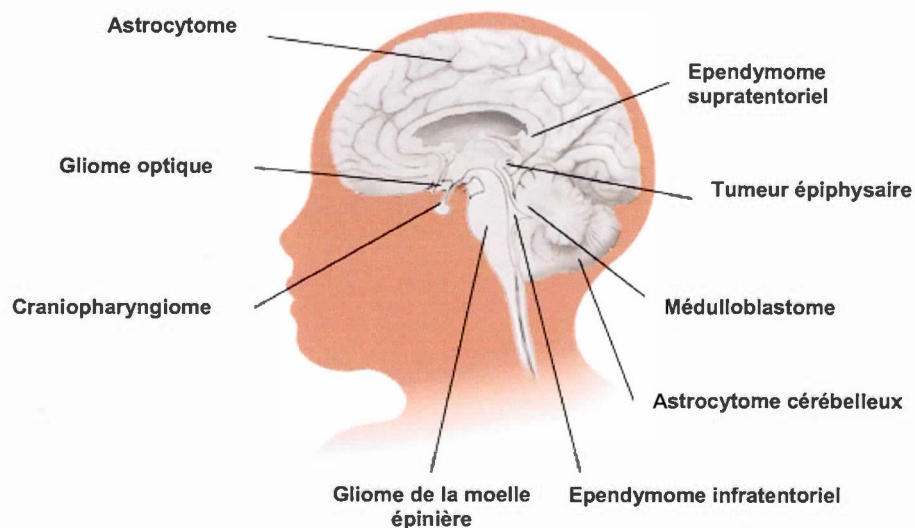


Figure 1.5 : Les tumeurs cérébrales chez l'enfant.

1.2.3. Les médulloblastomes.

1.2.3.1. Généralités.

Le médulloblastome est la plus fréquente des tumeurs cérébrales malignes de l'enfant. Il dérive du neurectoderme et se développe dans le cervelet (Becker and Hinton 1983). Il représente 15 à 20% de l'ensemble des tumeurs encéphaliques et 30% de celles localisées dans la fosse postérieure. Son incidence est environ de 5 à 10 cas sur un million d'enfants entre 0 et 14 ans. Le pic de fréquence se situe à 5 ans (Fondation Canadienne des tumeurs cérébrales). La cause du médulloblastome n'est généralement pas connue mais une prédisposition génétique est observée dans environ 10% des cas, notamment en cas de syndrome de Gorlin (Lacombe et al. 1990). D'autres syndromes génétiques ont aussi été associés au médulloblastome : le syndrome de Rubinstein-Taybi, l'anémie de Fanconi, le syndrome de Nijmegen Breakage, le syndrome de Turcot avec des mutations d'APC et le syndrome de Li-Fraumeni (McKinney 2005). Cette tumeur a un fort potentiel métastatique dans le système nerveux central (SNC), et parfois en dehors du SNC (Subramanian et al. 2002).

1.2.3.2. Les traitements des médulloblastomes.

L'exérèse chirurgicale est la première étape indispensable du traitement du médulloblastome. Elle doit conduire à porter le diagnostic et tendre à enlever la tumeur dans sa totalité en étant la moins traumatisante possible. Une exérèse totale est possible dans plus de 90 % des cas (Packer et al. 1999). L'irradiation du système nerveux central fait aussi partie intégrante du traitement standard (Paterson and Farr 1953). La radiothérapie est réalisée, dès que possible, après la chirurgie. Elle comprend systématiquement une irradiation du site primitif au niveau de la fosse cérébrale postérieure ainsi qu'une irradiation prophylactique de l'ensemble du système

nerveux central (moelle épinière et encéphale) (Bouffet et al. 1992). Pour les enfants les plus jeunes, les études actuelles cherchent à éviter l'irradiation du système nerveux central afin de diminuer les séquelles sur le système nerveux en développement.

Les médulloblastomes sont des tumeurs hautement chimiosensibles : cela est bien démontré dans les formes initiales (Pendergrass et al. 1987; Kovnar et al. 1990; Kalifa et al. 1992) ou dans les formes récidivantes (Lefkowitz et al. 1990; Gentet et al. 1994; Moghrabi et al. 1995). La chimiothérapie adjuvante est, par conséquent, efficace à tous les stades de la maladie. Enfin, en plus des thérapies classiques, l'immunothérapie a donné des résultats prometteurs dans le traitement des médulloblastomes (Geoerger et al. 2000). Cependant, la maladie et ses traitements induisent des séquelles à long terme, surtout endocriniennes et cognitives, en particulier pour les enfants traités à un jeune âge ou lorsque des complications de l'intervention chirurgicale se sont produites (Levisohn et al. 2000; Mulhern et al. 2004; Mulhern et al. 2005). Malgré tout, les protocoles actuels permettent la survie sans récurrence de 50 à 70% des patients. Celle-ci dépend de la qualité de l'exérèse et de l'existence d'une dissémination métastatique au moment du diagnostic.

1.3. L'ENDOTHÉLIUM VASCULAIRE.

1.3.1. Généralités.

L'endothélium vasculaire représente la monocouche de cellules endothéliales qui tapisse l'intérieur de tous les vaisseaux sanguins. L'endothélium est un tissu de type épithélial qui se différencie des autres épithéliums car il dérive du mésoderme embryonnaire (la plupart des autres dérivant de l'ectoderme ou de l'endoderme) (Lacaud et al. 2004). C'est aussi une membrane semi-perméable qui régule le trafic des leucocytes du sang vers les sites infectieux (Engelhardt and Wolburg 2004; Bazzoni 2006). Dans des situations pathologiques, les cellules tumorales franchissent elles aussi l'endothélium vasculaire ce qui leur permet de se disséminer dans tout l'organisme (métastases) en empruntant l'arbre vasculaire (Witz 2006).

1.3.2. La barrière hématoencéphalique.

Selon les organes, l'endothélium peut se spécialiser en vue de remplir une fonction spécifique de l'organe. Dans le rein, la lame basale est fenestrée, ce qui lui donne une fonction de filtre moléculaire (Camici 2005). Dans le système nerveux, au contraire, les cellules endothéliales sont reliées par des jonctions serrées empêchant les molécules de passer entre les cellules. L'endothélium est ici responsable des fonctions spécifiques de la barrière hématoencéphalique (Hawkins et al. 2006).

Au niveau de la barrière hématoencéphalique (BHE), les capillaires se différencient nettement des capillaires des autres tissus de l'organisme car ils sont bordés par une couche de cellules endothéliales qui adhèrent étroitement entre elles par des jonctions serrées de type zonula occludens (Engelhardt 2003) (Figure 1.6). Ces cellules forment une protection physique entre les cellules du cerveau (astrocytes, microglie, neurones, ...) et le sang. Il faut noter que la présence des jonctions serrées rend la diapédèse des leucocytes du sang vers le cerveau très difficile. Ces jonctions doivent s'assembler et se dissocier partiellement ou complètement au cours du

développement et au cours de divers processus physiologiques. Au regard du rôle majeur que jouent le calcium extracellulaire et les protéines kinases lors de l'établissement des jonctions serrées, ils ont été décrits comme des régulateurs de ces jonctions.

On trouve dans la jonction des cadhérines qui sont des protéines possédant un seul domaine transmembranaire (Wolburg and Lippoldt 2002). Les interactions dont elles sont l'objet dépendent du Ca^{++} et sont responsables des adhérences intercellulaires. Leur fonctionnement dépend de façon critique d'un groupe de protéines intracytoplasmiques appelées caténines qui interviennent comme des intermédiaires assurant le lien avec le cytosquelette (Wolburg and Lippoldt 2002). Ce jeu d'interactions avec le cytosquelette jouerait un rôle fondamental dans l'influence que les protéines d'adhérence exercent sur la jonction serrée. On soupçonne les cadhérines présentes dans les cellules endothéliales des vaisseaux cérébraux de jouer un rôle identique à celui des cellules épithéliales. En effet, la formation des jonctions serrées dans les cellules endothéliales du cerveau dépend du Ca^{++} (Wolburg and Lippoldt 2002).

La phosphorylation de résidus tyrosine des cadhérines pourrait aussi exercer un rôle régulateur de la perméabilité. On a montré qu'une tyrosine kinase dénommée lyn et appartenant à la famille src influence le phénotype des cellules endothéliales du cerveau (Achen et al. 1995). Récemment, il a également été trouvé que les cellules endothéliales traitées avec des inhibiteurs de tyrosine phosphatase augmentent la perméabilité des jonctions serrées (Lohmann et al. 2004). Cette augmentation est associée à une augmentation de la phosphorylation de la tyrosine des protéines intervenant dans les rapprochements cellulaires.

En ce qui concerne les jonctions communicantes de type gap, on en a observé entre les cellules endothéliales et les péricytes chez le rat. La signification fonctionnelle de ces jonctions est à ce jour inconnue (Nagasawa et al. 2006). On

pense que par l'intermédiaire de ces jonctions le péricyte pourrait réguler la prolifération de l'endothélium dans le cerveau en développement.

Malgré une importante capacité de synthèse, le cerveau a besoin pour son bon fonctionnement de nutriments apportés par le sang comme le glucose ou certains acides aminés. Comme la présence des jonctions serrées empêche le passage des molécules entre les cellules endothéliales des capillaires cérébraux (voie paracellulaire), toute substance devra, pour passer du sang vers le tissu cérébral, franchir la BHE par voie transcellulaire. Les molécules lipophiles de petite taille traversent facilement les membranes phospholipidiques, par simple diffusion, alors que les molécules polaires ou trop larges doivent utiliser des transporteurs spécifiques (Hawkins et al. 2006). Par ailleurs, il existe des systèmes d'efflux qui prennent en charge de nombreuses molécules lipophiles afin de les faire sortir du cerveau. C'est le cas de la glycoprotéine P ou P-gp (Cordon-Cardo et al. 1989).

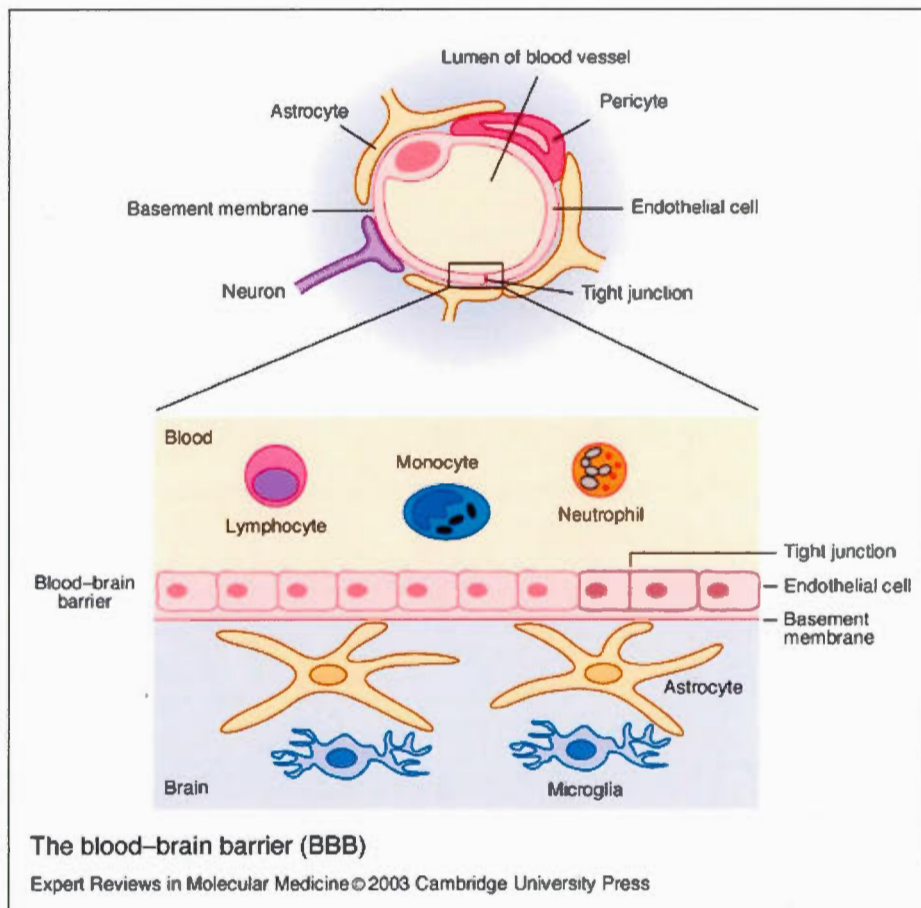


Figure 1.6 : La barrière hématoencéphalique.

1.3.3. La glycoprotéine P.

Les transporteurs membranaires, tout comme les systèmes enzymatiques tels que les cytochromes, sont des facteurs déterminants dans le devenir des médicaments dans l'organisme. La glycoprotéine P (P-gp) appartient à la superfamille des transporteurs ABC (*ATP-binding cassette*) et est probablement l'un des transporteurs les plus importants impliqués dans la biodisponibilité des xénobiotiques chez l'homme (Juliano and Ling 1976) (Figure 1.7). Elle peut transporter un grand nombre de molécules dont les structures sont très différentes les unes des autres (Tableau 1.2). La P-gp a été identifiée en 1986 comme l'une des causes majeures de chimiorésistance des cancers (Ueda et al. 1986). Elle est composée de 1 280 acides aminés et codée par un gène appelé *mdr1* (pour *multidrug resistance*) chez l'Homme et par deux orthologues *mdr-1a* et *mdr-1b* chez la souris et le rat (Juranka et al. 1989). La P-gp est N-glycosylée au niveau de sa troisième boucle cytoplasmique (Figure 1.8). Cette glycosylation joue un rôle majeur dans la fonction de transport de la P-gp (Kramer et al. 1995). Elle est présente en grandes quantités dans les cellules du foie, des tubules des reins et de la muqueuse du côlon, organes qui ont précisément un rôle d'élimination (Sugawara et al. 1988). On rencontre aussi une grande quantité de P-gp dans les endothéliums vasculaires du cerveau et du testicule (Sugawara et al. 1988; Beaulieu et al. 1997; Melaine et al. 2002).

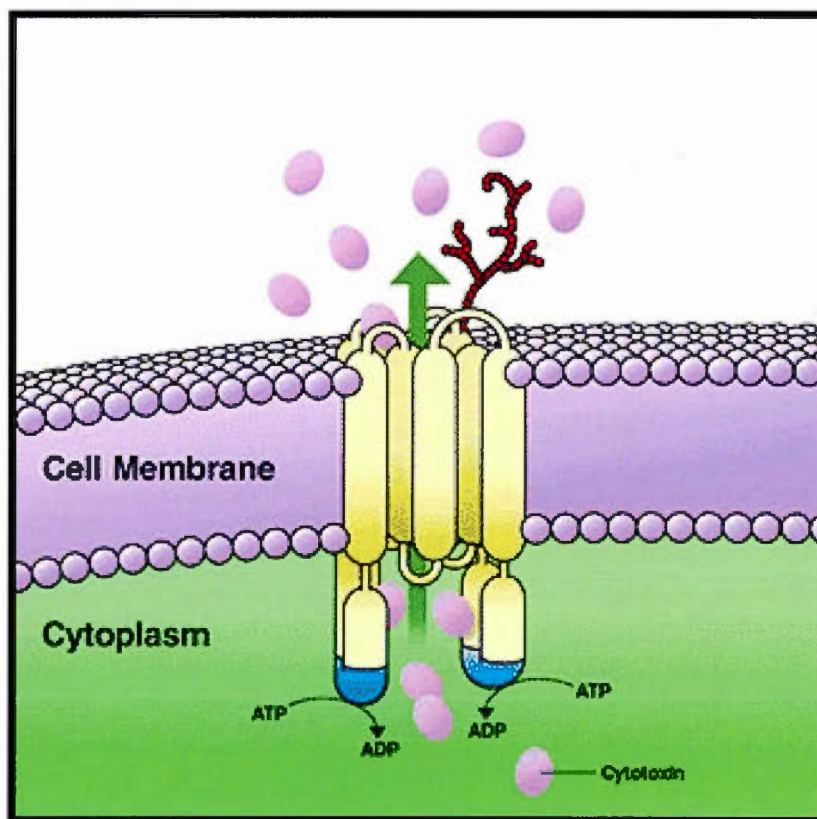


Figure 1.7 : Structure de la glycoprotéine P

La P-gp est considérée comme une protéine de réponse au stress cellulaire (Ledoux et al. 2003). Par conséquent, son expression peut être modulée par de nombreux stimuli. Ainsi, la P-gp est surexprimée en réponse à une baisse du pH intracellulaire, à un choc de température, aux agents cytotoxiques, aux médicaments anticancéreux et à l'irradiation UV (Szabo et al. 2000). La fonction de transport de la P-gp est aussi susceptible d'être inhibée par divers agents pharmacologiques comme le vérapamil ou la cyclosporine, qui ont été testés en clinique, en association avec la doxorubicine ou la vincristine, dans les tumeurs résistantes (Ishida et al. 1990; Zacherl et al. 1994) (Tableau 1.3).

Catégories	Molécules
Agents antitumoraux	Doxorubicine Taxol Vincristine Vinblastine Etoposide
Hormones stéroïdes	Aldostérone Cortisol Dexaméthasone
Agents antimicrobiens	Erythromycine Itraconazole Tétracycline Doxycycline
Opiïdes	Lopéramide Morphine
Drogues cardiaques	Digoxine Vérapamil Diltiazem
Immunosuppresseurs	Cyclosporine Tacrolimus
Divers	Ivermectin Phénothiazines Terfénadine Vécuronium

Tableau 1.2 : Quelques substrats de la P-gp (Mealey 2004)

Catégories	Molécules
Agents antidépresseurs	Fluoxétine Proxétine Millepertuis
Opioïdes	Méthazone Pentazocine
Agents antimicrobiens	Erythromycine Itraconazole
Drogues cardiaques	Vérapamil Amiodarone Carvédilol Quinidine Nicardipine
Immunosuppresseurs	Cyclosporine Tacrolimus
Divers	Jus de pamplemousse Bromocriptine Chlorpromazine Tamoxifène

Tableau 1.3 : Principaux inhibiteurs de la P-gp (Mealey 2004)

1.4. L'ANGIOGENÈSE TUMORALE.

1.4.1. Historique.

En 1971, un chirurgien américain, le Dr Judah Folkman, proposait un nouveau rôle pour les vaisseaux sanguins au cours de la croissance tumorale. Il émit l'hypothèse que la croissance des tumeurs était dépendante de l'angiogenèse (Folkman 1971). Cette hypothèse suggérait que les cellules tumorales et les cellules endothéliales vasculaires au sein d'une tumeur constituaient un système biologique intégré et que les cellules endothéliales pouvaient passer d'une phase de dormance à une phase de croissance rapide sous l'influence d'un ou de plusieurs facteurs chimiques diffusibles provenant des cellules tumorales (Folkman et al. 1963; Folkman et al. 1966; Folkman et al. 1971; Folkman 1972, 1976). Par conséquent, l'angiogenèse tumorale pouvait être une cible intéressante des thérapies anticancéreuses.

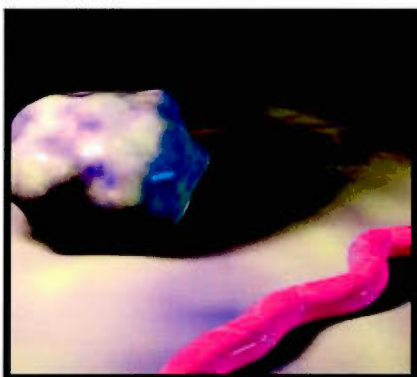
L'angiogenèse est un mécanisme de néovascularisation prenant naissance à partir d'un réseau capillaire préexistant (Folkman 1971). Elle se distingue de la vasculogenèse dans laquelle des précurseurs hémangioblastiques se différencient pour former des vaisseaux (Risau and Flamme 1995; Eichmann et al. 2002). Elle est particulièrement importante et indispensable au cours de nombreux processus physiologiques tels que le développement embryonnaire, l'implantation du placenta mais aussi pathologique, en particulier pour la croissance des tumeurs, le développement des métastases (Folkman 1972).

1.4.2. Angiogenèse et développement tumoral.

La vascularisation des tumeurs représente une étape critique au cours de la progression tumorale. En effet, la croissance des tumeurs et l'expansion métastatique sont strictement contrôlées par la néovascularisation. Les cellules tumorales traversent, dans leur histoire, deux phases. La première est dite dormante, la deuxième angiogénique et invasive. Le passage entre les deux est contrôlé par un relais moléculaire (dit « *switch* » angiogénique) (Tosetti et al. 2002; Bergers and Benjamin 2003). Les tumeurs solides dont la taille est inférieure à un millimètre cube ne sont pas vascularisées, ce qui signifie que pour que la tumeur croisse et s'étende, elle doit obtenir l'oxygène et les nutriments véhiculés par les vaisseaux sanguins et être débarrassée de ses déchets métaboliques. Au-delà de cette taille d'un millimètre cube, l'oxygène et les nutriments ont de la difficulté à atteindre les cellules au centre de la tumeur, ce qui provoque un état d'hypoxie cellulaire qui déclenche l'angiogenèse tumorale (Richard et al. 1999).

Dans un premier temps, les cellules tumorales libèrent des molécules qui envoient des signaux aux tissus endothéliaux sains environnants. Ces signaux activent la transcription de certains gènes codant pour des protéines qui favorisent la formation de nouveaux vaisseaux sanguins (Folkman 1971). La formation de ces nouveaux vaisseaux sanguins est un facteur crucial pour la progression des tumeurs puisqu'elle favorise le passage d'un état de multiplication raisonnée des cellules à un état de prolifération incontrôlée, caractéristique des cellules cancéreuses (Figure 1.8). La néovascularisation influence aussi la dissémination des cellules cancéreuses dans l'ensemble de l'organisme, puis la formation de métastases (Mahadevan and Hart 1990). On a déterminé que le niveau de vascularisation d'une tumeur solide est un excellent indicateur de son potentiel métastatique et de la probabilité de survie des patients (Takahashi et al. 1995; Takahashi et al. 1997). Ainsi, plus l'expression du VEGF, un facteur proangiogénique, est importante dans les cellules tumorales, plus le

nombre de vaisseaux sanguins augmente au sein de la tumeur (Figure 1.9) et plus l'espérance de vie des patients est courte (Figure 1.10).



Une tumeur initiale primitive dormante non vascularisée peut rester asymptomatique car les cellules se multiplient à un rythme à peu près équivalent à celui de l'apoptose. Si la multiplication cellulaire s'accroît ou si une résistance à l'apoptose s'installe, les cellules au centre de la tumeur deviennent hypoxiques et fabriquent du VEGF et du FGF (Richard et al. 1999).



Sous l'action de facteurs proangiogéniques issus de la tumeur, les cellules endothéliales qui tapissent les vaisseaux existants synthétisent des enzymes protéolytiques (métalloprotéinases, collagénases) qui vont rompre la membrane basale endothéliale existante, prolifèrent et migrent alors vers la source du stimulus angiogénique. Elles forment ensuite de nouveaux vaisseaux sanguins qui vascularisent la tumeur (Folkman 1971).



Les vaisseaux sanguins apportent à la tumeur l'oxygène et les nutriments nécessaires à sa croissance. Par conséquent, grâce à l'angiogenèse tumorale, la tumeur peut poursuivre sa croissance. De plus, ces vaisseaux représentent une porte de sortie pour les quelques 100000 cellules tumorales qui se détachent de la tumeur tous les jours. En empruntant la circulation systémique, ces cellules pourront former de nouvelles tumeurs à distance dans l'organisme : les métastases (Mahadevan and Hart 1990).

Figure 1.8 : Les différentes étapes de l'angiogenèse

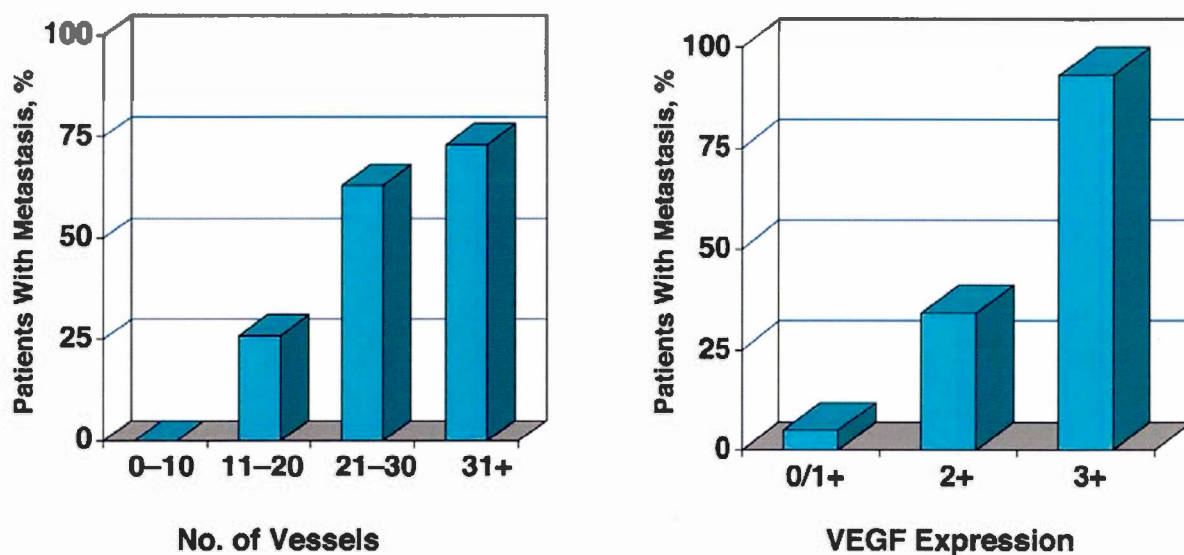


Figure 1.9 : Corrélation entre l'expression du VEGF, la densité vasculaire et le potentiel métastatique des tumeurs.
(Takahashi et al. 1995).

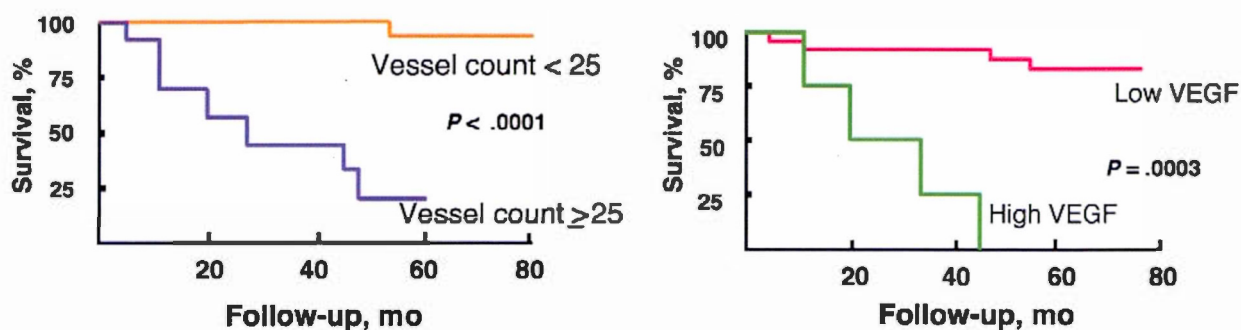


Figure 1.10 : Corrélation entre l'expression du VEGF, la densité vasculaire et la survie des patients.
(Takahashi et al. 1997).

1.4.3. Les mécanismes de l'angiogenèse.

Trois mécanismes fondamentaux de l'angiogenèse ont été identifiés : le « sprouting », l'intussusception et la cooption. Au cours du « sprouting », les cellules endothéliales sont activées, ce qui conduit à la dégradation de la membrane basale et de la matrice extracellulaire environnante. La migration orientée des cellules endothéliales est suivie d'une phase de prolifération, puis d'une différenciation en structure capillaire (de Waal and Leenders 2005) (Figure 1.8). L'angiogenèse intussusceptive est caractérisée par un élargissement et une séparation de vaisseaux déjà formés (Burri and Djonov 2002; Djonov et al. 2003; Burri et al. 2004). Enfin, il existe des évidences cliniques et expérimentales que des tumeurs grandissent en utilisant des vaisseaux déjà présents, une condition que l'on nomme la cooption vasculaire. Par exemple, jusqu'à 16% des carcinomes pulmonaires non à petites cellules grandissent autour de structures capillaires alvéolaires, sans évidence d'angiogenèse par « sprouting » (Pezzella et al. 1997). Ce mécanisme de cooption est aussi observé dans les tumeurs cérébrales (Rubenstein et al. 2000). Les mécanismes moléculaires de ces différents modes d'angiogenèse sont complexes, puisqu'ils mettent en jeu de nombreux signaux extra et intracellulaires.

1.4.4. Les régulateurs de l'angiogenèse.

Au cours de ces dernières années, il est devenu clair que l'angiogenèse n'est pas contrôlée par un seul facteur, mais par une balance d'inducteurs et d'inhibiteurs produits par les cellules normales ou tumorales (Milkiewicz et al. 2006) (Tableau 1.4). Parmi les régulateurs clés de l'angiogenèse figurent entre autres les « *Vascular Endothelial Growth Factors* (VEGF) » et leurs récepteurs (Byrne et al. 2005; Ceballos et al. 2006), les « *Fibroblast Growth Factors* (FGF) » et leurs récepteurs (Presta et al. 2005), les angiopoïétines (Reiss et al. 2005), Tie-1, Tie-2 (Thurston 2003), les métalloprotéases et leurs inhibiteurs (Lakka et al. 2005; Rundhaug 2005; Stetler-Stevenson and Seo 2005), les lysophospholipides (sphingosine-1-phosphate et

acide lysophosphatidique) (English et al. 2002) et les activateurs du plasminogène (Rakic et al. 2003). D'autre part, des régulateurs négatifs endogènes comme la thrombospondine (Armstrong and Bornstein 2003), l'angiostatine (O'Reilly 1997), l'endostatine (Cao 1998), et le facteur plaquettaire-4 (Bikfalvi 2004) ont aussi été décrits. En dehors de ces facteurs solubles, les intégrines jouent un rôle considérable dans ce processus en contrôlant l'adhésion et la migration des cellules endothéliales grâce aux interactions avec la matrice extracellulaire (Stupack and Cheresch 2002; Serini et al. 2006).

Catégories	Molécules
Stimulateurs de l'angiogenèse	Angiogénine.
	Angiopoietine-1.
	Facteur de croissance des fibroblastes: acide (aFGF) et basique (bFGF).
	Follistatine.
	Facteur de croissance des granulocytes (GCSF).
	Facteur de croissance des hépatocytes (HGF).
	Interleukine-8 (IL-8).
	Leptine.
	Facteur de croissance placentaire.
	Facteur de croissance plaquettaire-BB (PDGF-BB).
	Pléiotrophine (PTN).
	Progranuline.
	Proliférine.
	Facteur de croissance transformant-alpha (TGFalpha)
	Facteur de croissance transformant -beta (TGFbeta)
	Facteur de nécrose tumorale-alpha (TNF-alpha)
	Facteur de croissance de l'endothélium vasculaire (VEGF) / facteur de perméabilité vasculaire (VPF)
	Sphingosine-1-phosphate (S1P)
	Acide lysophosphatidique (LPA)

Inhibiteurs de
l'angiogenèse

Angioarrestine
 Angiostatine (fragment du plasminogène)
 Antithrombine antiangiogénique III
 Inhibiteur dérivé du cartilage (CDI)
 Endostatine (fragment du collagène XVIII)
 Fragment de la fibronectine
 Héparinases
 Fragment de l'héparine hexasaccharide
 Gonadotrophine chorionique humaine (hCG)
 Interféron alpha/beta/gamma
 Protéine induite par l'interféron (IP-10)
 Interleukine-12
 Kringle-5 (fragment du plasminogène)
 Inhibiteurs tissulaires des métalloprotéases (TIMPs)
 2-méthoxyestradiol
 Inhibiteurs des ribonucléases placentaires (PRI)
 Inhibiteurs des activateurs du plasminogène (PAI)
 Facteur plaquettaire-4 (PF4)
 Fragment de 16kD de la prolactine
 Rétinoides
 Tétrahydrocortisol-S
 Thrombospondine-1 (TSP-1)
 Facteur de croissance transformant-beta (TGF- β)
 Vasculostatine
 Vasostatine (calreticulin fragment)

Tableau 1.4 : Régulateurs endogènes de l'angiogenèse

(Angiogenesis Foundation).

1.5. LES INTÉGRINES

1.5.1. Généralités

Les intégrines sont les membres d'une famille de récepteurs impliqués dans les interactions adhésives entre cellules et entre les cellules et la matrice extracellulaire (MEC) (Buck and Horwitz 1987). Les intégrines jouent un rôle majeur dans la migration cellulaire, l'organisation tissulaire, la différenciation, la prolifération et l'apoptose (Sheppard 2000; Pozzi and Zent 2003). Ces protéines sont aussi impliquées dans diverses pathologies humaines, comme les thromboses, l'inflammation, l'invasion et les métastases dans le cancer, l'ostéoporose, ou encore, les infections bactériennes et parasitaires (Cheresh 1991; Lione et al. 2005; Zhao et al. 2005).

1.5.2. Structure des intégrines

Les intégrines sont des hétérodimères formés d'une chaîne α et d'une chaîne β liées de façon non covalente (Humphries 2000) (Figure 1.11). Il a été décrit 18 chaînes α et 8 chaînes β qui peuvent se combiner en 24 hétérodimères différents (Figure 1.12). Les intégrines de type $\beta 1$ et $\beta 3$ sont exprimées par la plupart des types cellulaires et interagissent principalement avec la matrice extracellulaire (MEC). De plus, les intégrines de type $\beta 1$ médient surtout des interactions avec des macromolécules des tissus connectifs telles que la fibronectine, les collagènes et les laminines (Hemler et al. 1990), alors que les intégrines de type $\beta 3$ sont plutôt associées aux mécanismes adhésifs avec des composants «vasculaires» tels que la fibronectine, le fibrinogène, la vitronectine, la thrombospondine et le facteur de von Willebrand (Suehiro and Plow 1997). Les intégrines de type $\beta 2$ sont uniquement exprimées dans les leucocytes et sont impliquées dans des interactions cellulaires hétérotypiques avec les CAM-Ig et des interactions leucocytes-cellules endothéliales vasculaires (Bosman 1993).

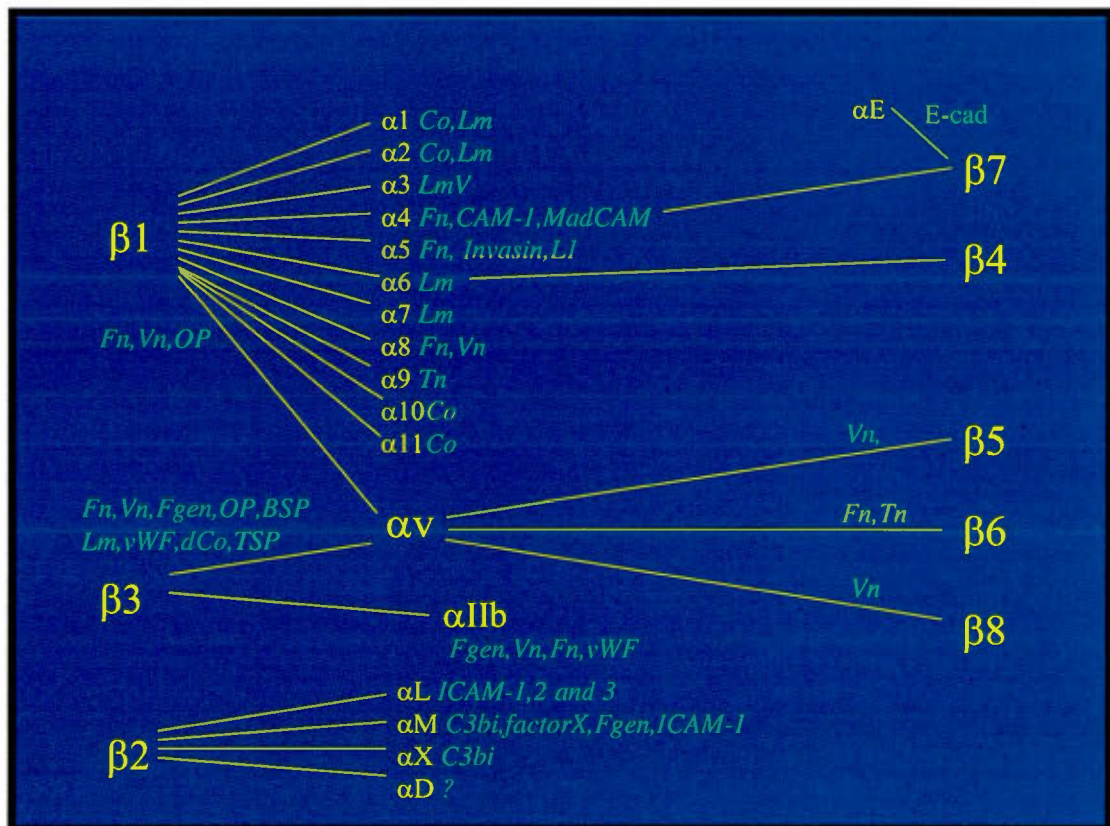


Figure 1.12 : Les différentes intégrines (Hynes 1987)

Fn : fibronectine; *Vn* : vitronectine; *Lm* : laminine; *Co*: collagène; *vWF*: facteur de von Willebrand; *Fgen*: fibrinogène; *TSP*: thrombospondine; *OP* : ostéopontine; *Tn* : ténectine; *E-cad* : E-cadhérine; *BSP* : sialoprotéine de l'os; *factor X* : facteur X de la coagulation; *C3bi* : composant C3 du complément inactivé.

1.5.3. Les interactions intégrines-ligands

La spécificité de liaison des intégrines à leurs ligands met deux propriétés en évidence. Premièrement, certaines intégrines sont capables de se lier à des ligands dont les structures sont très dissemblables. Par exemple, l'intégrine $\alpha V\beta 3$ peut lier des protéines aussi différentes que la fibronectine, la vitronectine ou le fibrinogène. Deuxièmement, certains ligands peuvent interagir avec plusieurs intégrines. Par exemple, la fibronectine se lie aux intégrines $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha IIb\beta 3$, $\alpha 4\beta 7$, $\alpha V\beta 1$, $\alpha V\beta 3$, $\alpha V\beta 5$ et $\alpha V\beta 6$ (Tableau 1.5).

Ligands	Intégrines $\beta 1$	Intégrines αV	Autres
Collagènes	$\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$		
Laminines	$\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$		$\alpha 6\beta 4$
Fibronectine	$\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$	$\alpha V\beta 1$, $\alpha V\beta 3$, $\alpha V\beta 6$, $\alpha V\beta 8$	$\alpha 4\beta 7$, $\alpha IIb\beta 3$
Vitronectine		$\alpha V\beta 1$, $\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha V\beta 8$	$\alpha IIb\beta 3$
Fibrinogène		$\alpha V\beta 3$	$\alpha IIb\beta 3$
Ténascine	$\alpha 2\beta 1$, $\alpha 8\beta 1$, $\alpha 9\beta 1$		

Tableau 1.5 : Liste des intégrines liant les principales protéines de la MEC.

(Hynes 1987)

L'un des facteurs qui détermine la liaison des intégrines à certaines protéines de la MEC ou à d'autres récepteurs (tels que les protéines d'adhérence cellulaire) est la présence dans ces ligands de motifs de reconnaissance des intégrines. L'identification moléculaire de ces sites de liaison aux intégrines a nécessité la genèse de fragments de protéines de la MEC, puis l'analyse de ces fragments dans des tests d'adhérence cellulaire. Par exemple, le motif RGD a été tout d'abord trouvé dans le domaine central de liaison de la fibronectine (Pierschbacher and Ruoslahti 1984; Yamada and Kennedy 1984, 1985), puis dans de nombreuses autres protéines de la MEC, telles que le fibrinogène, les collagènes ou le facteur de von Willebrand (Humphries 1990). De façon générale, et malgré la grande diversité des ligands aux intégrines, ils ont en commun un résidu d'acide aspartique ou d'acide glutamique généralement exposé dans une boucle flexible et dont la présence est critique pour la reconnaissance par l'intégrine (Jones et al. 1995; Leahy et al. 1996; Bella et al. 1998; Casanovas et al. 1998). Ces résidus sont présents dans des motifs de reconnaissance trouvés en une ou plusieurs copies dans le ligand. Les motifs à acide aspartique (RGD, LVD, KGD, RTD et KQAGD) peuvent se lier à la majorité des intégrines (Ruoslahti 1996). Cependant, neuf intégrines préfèrent se lier à des ligands possédant des motifs de reconnaissance basés sur l'acide glutamique (Michishita et al. 1993).

Le second facteur qui affecte les interactions intégrines – ligands est l'effet des cations divalents. Les résidus acide aspartique ou glutamique présents dans les ligands forment un complexe ternaire avec l'intégrine et les cations divalents (Xiong et al. 2001; Xiong et al. 2002). Les cations divalents impliqués dans ces complexes sont le magnésium (Mg^{2+}), le manganèse (Mn^{2+}) et le calcium (Ca^{2+}). Alors que le Mg^{2+} et le Mn^{2+} permettent une liaison physiologique dans une large gamme de concentration, le Ca^{2+} semble requérir une concentration de l'ordre du micromolaire, identique à celle du plasma (Mould et al. 1995). De plus, le Ca^{2+} inhibe généralement les interactions intégrines-ligands.

Le troisième facteur important est le niveau d'activation des intégrines. Ceci semble jouer un rôle majeur dans la reconnaissance des motifs présents dans les ligands. Par exemple, des interactions de faible affinité médiées par des regroupements d'intégrines sont responsables de l'attachement des cellules à leur substrat ou au roulement des leucocytes (Grabovsky et al. 2000; Feigelson et al. 2001). Des adhérences fortes nécessitent, par contre, des interactions de haute affinité (Grabovsky et al. 2000; Feigelson et al. 2001; Litvinov et al. 2002). Les liaisons de haute affinité des intégrines à un ligand requièrent une modification conformationnelle du récepteur d'un niveau de basse affinité vers un niveau de haute affinité et peuvent être associées à une activation des intégrines (Figure 1.13). Cette modification est réalisée naturellement suite à l'interaction avec les cations divalents ou par une signalisation «*inside-out*» (Arnaout 2002; Schwartz and Ginsberg 2002; Calderwood et al. 2003; Qin et al. 2004). Ce signal «*inside-out*» est un signal intracellulaire transmis aux intégrines qui déclenche leur changement de conformation et augmente leur affinité pour les ligands (Figure 1.14). Lors de l'activation de l'intégrine, le domaine extracellulaire se détend, faisant se déplacer le site de liaison au ligand loin de la membrane (Beglova et al. 2002; Takagi et al. 2002; Qin et al. 2004). Ceci est accompagné d'autres réarrangements dans le site de liaison, augmentant l'affinité de l'intégrine pour son ligand pour son ligand (Figure 1.14).

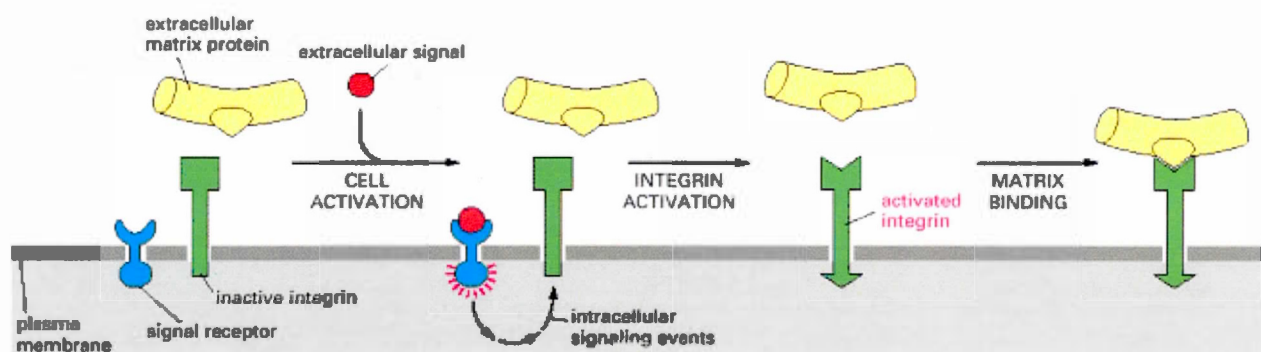


Figure 1.13 : Principe d'activation des intégrines.

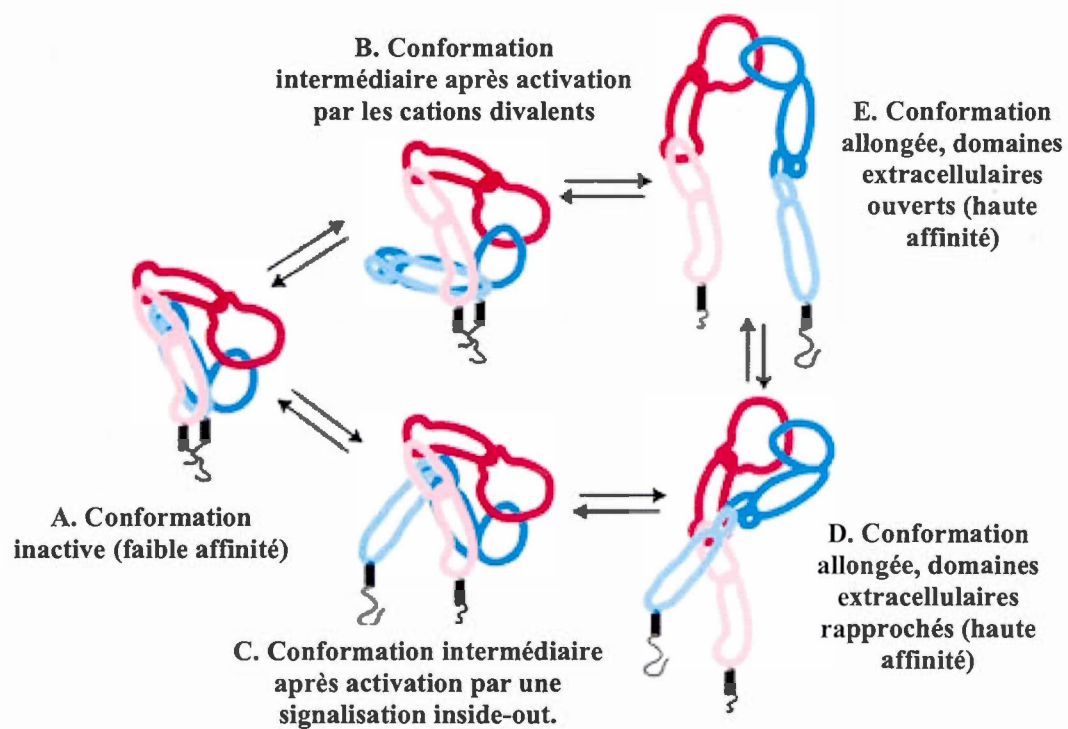


Figure 1.14 : Modifications de la structure quaternaire des intégrines au cours de l'activation. (Takagi et al. 2002)

1.5.4. Les intégrines et l'adhérence cellulaire.

L'adhérence des cellules à la matrice extracellulaire est fondamentale pour l'homéostasie cellulaire. Les signaux, transduits via la famille des intégrines, activent des cascades intracellulaires de signalisation qui modulent les réseaux du cytosquelette et l'expression génétique requis au cours de l'embryogenèse et lors de certains processus pathologiques comme la survie cellulaire, la prolifération ou l'apoptose (Figure 1.15) (Hynes 1992; Kornberg and Juliano 1992).

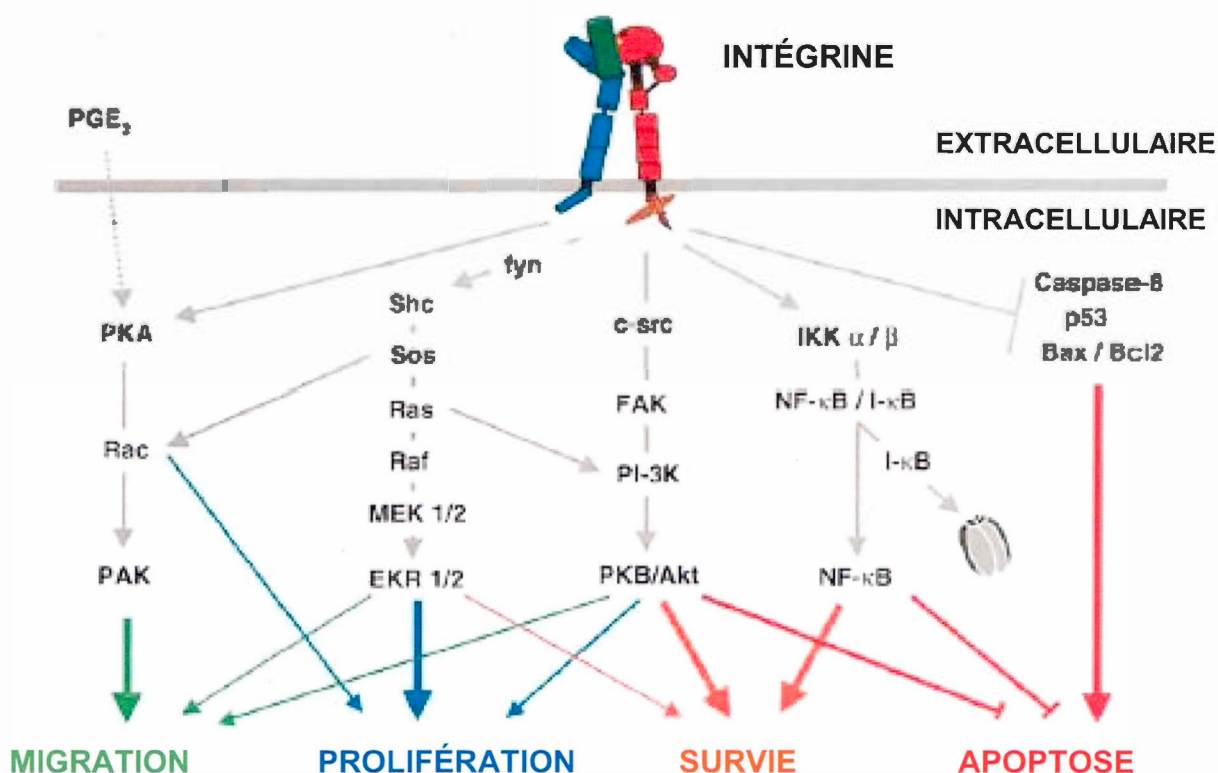


Figure 1.15 : Signalisation via les intégrines (Ruegg and Mariotti 2003)

Lorsque la cellule commence à s'étaler, de petites structures ponctiformes, les complexes focaux, apparaissent à l'extrémité des lamellipodes (Small et al. 2002). Ces complexes focaux sont à l'origine des contacts focaux (ou adhérences focales). (Geiger and Bershadsky 2001; Geiger et al. 2001). Les adhérences focales permettent l'ancrage des filaments d'actine à la MEC et une adhérence forte (Critchley et al. 1999) (Figure 1.16). Au niveau des contacts focaux, de nombreuses protéines intracytoplasmiques assurent le lien entre le domaine cytoplasmique des intégrines et les microfilaments d'actine. On peut citer, par exemple, la taline, la vinculine, la paxilline, l' α -actinine ou la zyxine (Figure 1.16). Les contacts focaux stabilisés et matures servent de point d'origine à la translocation d'intégrines des protrusions membranaires vers le corps cellulaire. Cette translocation conduit à la formation de sites d'adhérence fibrillaire (Pankov et al. 2000; Geiger et al. 2001). Ces structures créent des tensions sur la fibronectine notamment, exposant des sites cryptiques de polymérisation, ce qui favorise la fibrillogenèse (Pankov et al. 2000; Ohashi et al. 2002). Enfin, après accumulation d'une MEC, la cellule se trouve dans un environnement tridimensionnel. Les contacts focaux, stables et rigides, se transforment alors en un autre type de sites d'adhérence, appelé adhérence-3D, dont la composition et le patron de phosphorylation des protéines sont différents des autres types d'adhérence (Cukierman et al. 2001).

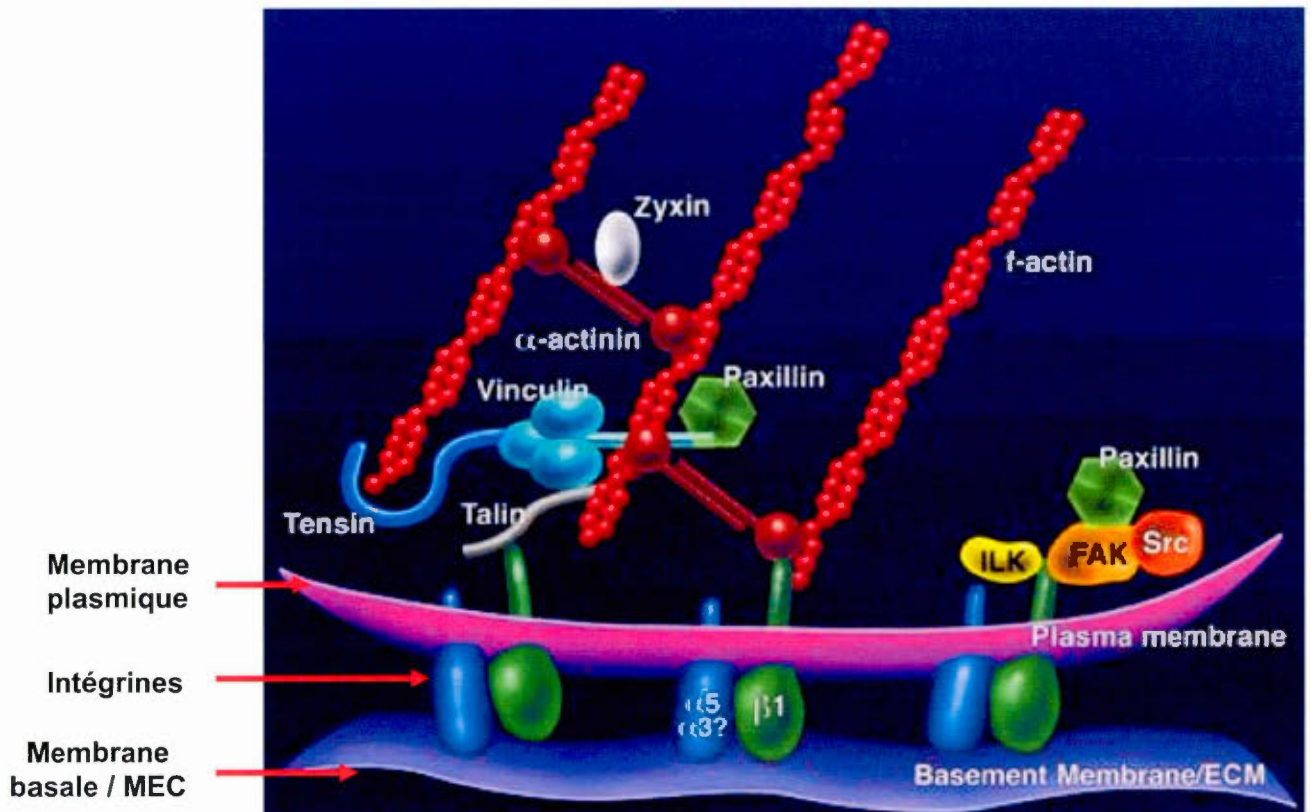
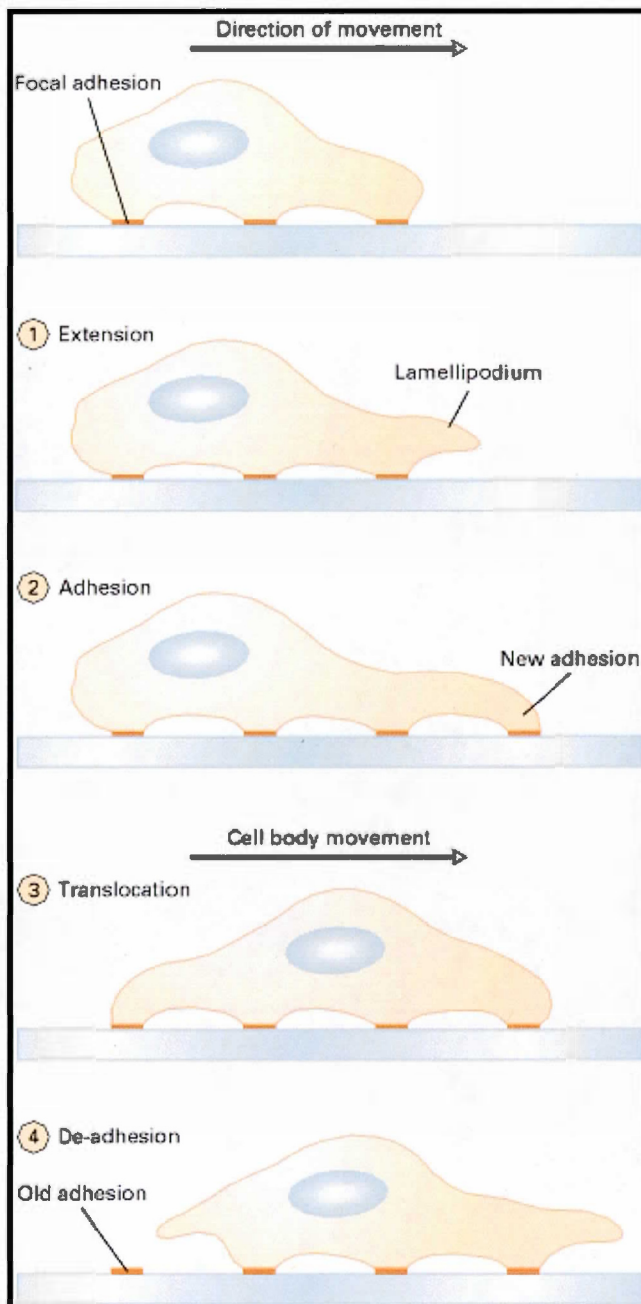


Figure 1.16 : Complexe moléculaire des contacts focaux (Bailly 2003)

1.5.5. Les mécanismes cellulaires de la migration.

La migration d'une cellule est un phénomène complexe qui peut être partagée en quatre phases distinctes : l'adhérence aux substrats, l'étalement, la traction et enfin la rétraction de l'arrière de la cellule (Kirkel et al. 2004). Comme exposé précédemment, l'interaction entre les intégrines et leurs substrats (les protéines de la MEC) permet l'adhérence cellulaire. Pour migrer, la cellule doit ensuite transformer ce mécanisme d'adhérence en mouvement et la coordination entre ces deux processus dépend principalement du cytosquelette d'actine et des microtubules (Brakebusch and Fassler 2003; Li et al. 2005).

Divers modèles mathématiques ont été élaborés pour comprendre la relation qui existe entre l'adhérence et la migration (Palecek et al. 1997). On a ainsi pu déterminer que l'adhérence augmente en fonction de la concentration en substrats, la quantité d'intégrines à la surface cellulaire et le niveau d'activation des intégrines. Ainsi, avec de faibles concentrations de substrats ou d'intégrines, la migration est lente, car l'adhérence trop faible, ne permet pas d'exercer les forces de tractions suffisantes. Il faut donc que l'adhérence augmente, pour que la vitesse de migration augmente. Cependant, si l'adhérence est trop importante, elle immobilise la cellule (Li et al. 2005). Des signaux «*outside-in*» et des signaux «*inside-out*» régulent la migration cellulaire en modulant l'affinité des intégrines pour leurs substrats (Coppolino and Dedhar 2000). L'autre processus favorisant la migration, implique le recyclage des intégrines de l'arrière vers l'avant de la cellule par des transports vésiculaires le long des microtubules (Caswell and Norman 2006). La capacité des cellules à migrer dépend donc de leur capacité à créer des interactions entre la cellule et le substrat au front de migration et à rompre les contacts établis à l'arrière lorsqu'ils ne sont plus nécessaires à la traction (Figure 1.17).



Dans un premier temps, la cellule émet des prolongements cytoplasmiques appelés lamellipodes dans le sens de la migration.

Les prolongements cytoplasmiques forment de nouvelles adhérences focales impliquant les intégrines.

Le corps cellulaire translate dans la direction de la migration grâce à la traction exercée sur les adhérences nouvellement formées.

La cellule rompt ensuite les contacts focaux situés à l'arrière de la cellule et reprend ce cycle d'adhérence et de désadhérence.

Figure 1.17 : Les différentes étapes de la migration cellulaire.

1.5.6. Intégrines et angiogenèse.

$\alpha V\beta 3$ fut la première intégrine directement associée à l'angiogenèse (Varner and Cheresh 1996; Eliceiri and Cheresh 2000). $\alpha V\beta 3$ est fortement exprimée dans les cellules endothéliales présentes au niveau des sites de cicatrisation ou dans les tumeurs malignes. Cependant, son expression est généralement faible dans les cellules endothéliales quiescentes (Gingras et al. 1995). Les antagonistes pharmacologiques de l'intégrine $\alpha V\beta 3$ inhibent l'angiogenèse (Varner and Cheresh 1996). Des molécules antagonistes de cette intégrine (EMD121974 ou Cilengitide) et la forme humanisée de l'anticorps bloqueur de fonction LM609 (Vitaxin) sont actuellement testés cliniquement pour mesurer leurs effets antitumoraux (Tucker 2003). Paradoxalement, l'analyse de souris n'exprimant pas le gène codant pour la sous-unité αV des intégrines a montré que cette intégrine était plutôt antiangiogénique (Bader et al. 1998). Il semble que ces résultats opposés soient liés au rôle important joué par les intégrines αV dans l'activation de l'apoptose cellulaire via la caspase-8. En effet, la liaison d'un antagoniste à cette intégrine déclencherait l'apoptose des cellules endothéliales en activant la caspase, et donc serait antiangiogénique, alors que l'absence de cette intégrine dans les souris $\alpha V^{-/-}$ rendrait les cellules endothéliales immortelles, d'où une prolifération endothéliale massive faisant penser à un processus angiogénique (Sheppard 2002). D'autres intégrines sont aussi impliquées dans le processus angiogénique. L'intégrine $\alpha 5\beta 1$ est essentielle à la vasculogenèse et est surexprimée au cours de l'angiogenèse (Kim et al. 2000b; Francis et al. 2002). Elle régulerait notamment la fonction de l'intégrine $\alpha V\beta 3$ durant la migration (Kim et al. 2000a). Certaines autres intégrines de la famille $\beta 1$, telles que les intégrines $\alpha 1\beta 1$ et $\alpha 2\beta 1$ jouent elles aussi un rôle majeur dans le processus angiogénique (Senger et al. 1997).

1.6. LES MÉTALLOPROTÉASES DE MATRICE.

Les MMPs sont une famille d'enzymes dont l'activité est dépendante d'un atome de zinc. Elles peuvent être classées en deux groupes : les MMPs sécrétées et les MMPs associées à la membrane (MT-MMPs) (Pepper 2001). Les MMPs sécrétées comprennent les collagénases (MMP-1, MMP-8, MMP-13), les gélatinases (gélatinase A, MMP-2 ; gélatinase B, MMP-9), les stromélysines (stromélysine-1, MMP-3 ; stromélysine-2, MMP-10 ; stromélysine-3, MMP-11) et les autres MMPs (MMP-7 et MMP-12). Six métalloprotéases de type membranaires (MT-MMPs) ont été identifiées (de MT1-MMP à MT6-MMP) (Zucker et al. 2003) (Figure 1.18). Ces enzymes sont sécrétées sous la forme de pro-enzyme (zymogène) dont l'activation se produit dans le compartiment extracellulaire ou au contact de la membrane plasmique pour MMP-2 (Murphy et al. 1999). De par sa localisation membranaire, MT1-MMP est impliquée dans le processus de dégradation matricielle localisé au niveau de la surface cellulaire, et surtout dans l'activation des précurseurs d'autres protéases comme la MMP-2 (Itoh and Seiki 2006). En plus de l'activation des zymogènes, l'activité MMP est régulée par les inhibiteurs tissulaires des MMPs, les TIMPs. Quatre inhibiteurs de MMPs ont été identifiés et forment des complexes covalents avec toutes les MMPs actives (Noel et al. 2004). L'expression des MMPs est stimulée par des facteurs de croissance, des cytokines, des promoteurs de tumeurs et les interactions intercellulaires ou cellule-MEC (Murphy and Docherty 1992). Plusieurs études ont montré l'importance des MMPs dans la pathogenèse des cancers et au cours de l'angiogenèse (Stetler-Stevenson 2001). Pour ces raisons, différentes approches thérapeutiques sont développées pour inhiber l'activité des MMPs. Les inhibiteurs naturels (TIMP-1 et -2) ou synthétiques (batimastat, marimastat), la calphostine (inhibiteur de la PKC), ou encore des vecteurs antisens et des ribozymes ciblant les MMPs se sont montrés efficaces dans des systèmes *in vitro* ou dans des modèles animaux (Lakka et al. 2005).

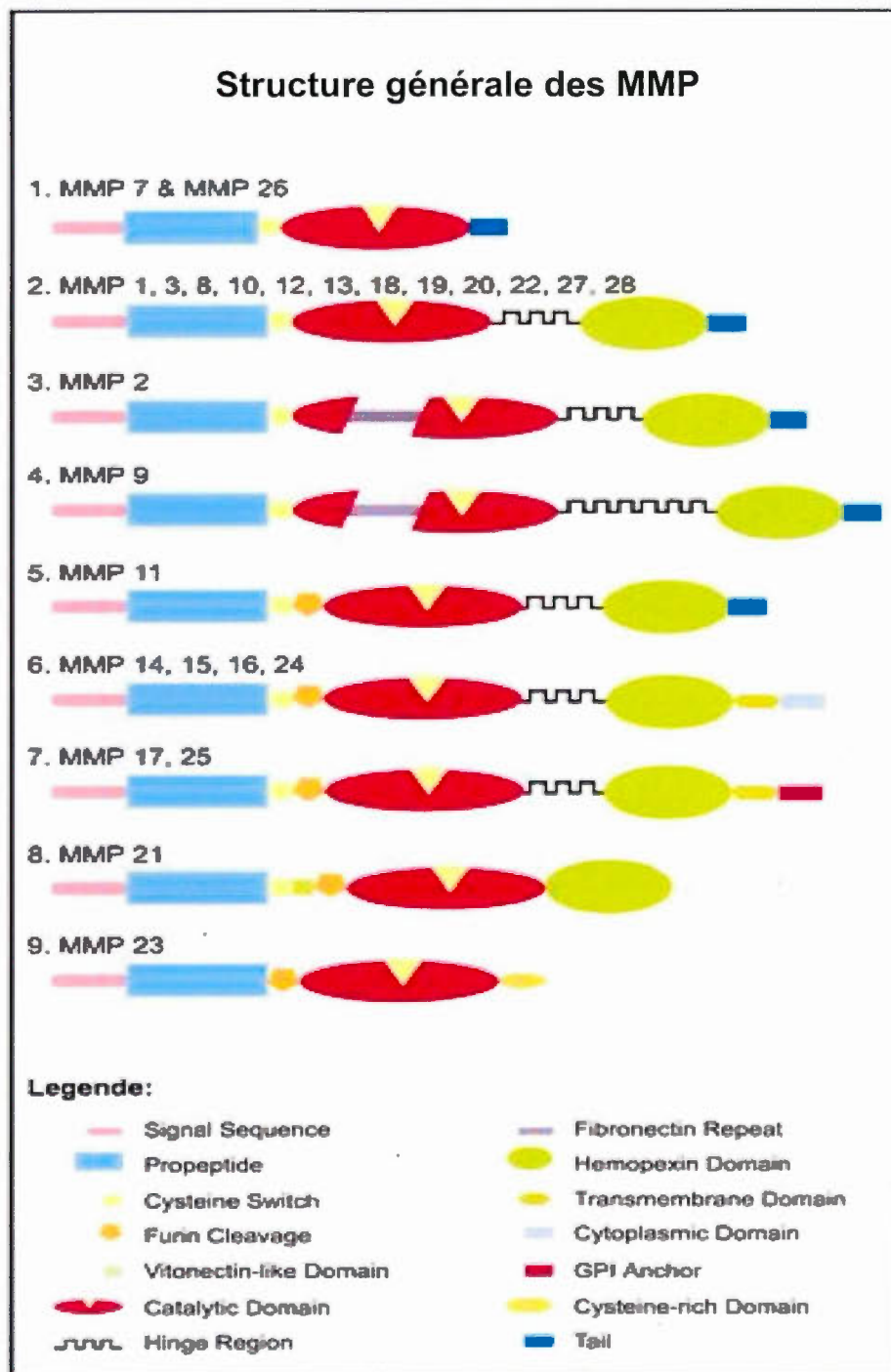


Figure 1.18 : Structure des métalloprotéases de matrice. (Lafleur et al. 2003)

1.7. LA SPHINGOSINE-1-PHOSPHATE

1.7.1. Généralités

La sphingosine-1-phosphate ou S1P est un sphingolipide polaire stocké dans les plaquettes et libéré au cours de l'activation plaquettaire (Igarashi and Yatomi 1998) (Figure 1.19). De nombreux autres types cellulaires sécrètent la S1P. C'est le cas des monocytes, des cellules mastocytaires, des cellules endothéliales, mais aussi des érythrocytes et des glioblastomes (Maceyka et al. 2005a; Van Brocklyn et al. 2005). La concentration de S1P dans le plasma varie entre 0.1 et 0.2 μM . Dans le sang, la S1P se retrouve principalement sous une forme liée à l'albumine, mais une petite quantité se retrouve aussi sous une forme libre (Igarashi and Yatomi 1998). La S1P est sécrétée en réponse à divers facteurs de croissance et cytokines et peut agir de façon paracrine et/ou autocrine (Rosen and Goetzl 2005). Plusieurs récepteurs membranaires de haute affinité, liés aux petites protéines G, lient la S1P extracellulaire et activent diverses voies de transduction intracellulaires. Ces récepteurs ont une distribution tissulaire très large et se retrouvent aussi bien dans les tissus embryonnaires que chez l'adulte (Rosen and Goetzl 2005). La S1P affecte de nombreux processus biologiques tels que la croissance cellulaire, la différenciation, la migration et l'apoptose et peut aussi jouer un rôle majeur dans certaines conditions physiopathologiques telles que l'athérosclérose ou le cancer (Spiegel and Milstien 2003; Hla 2004).

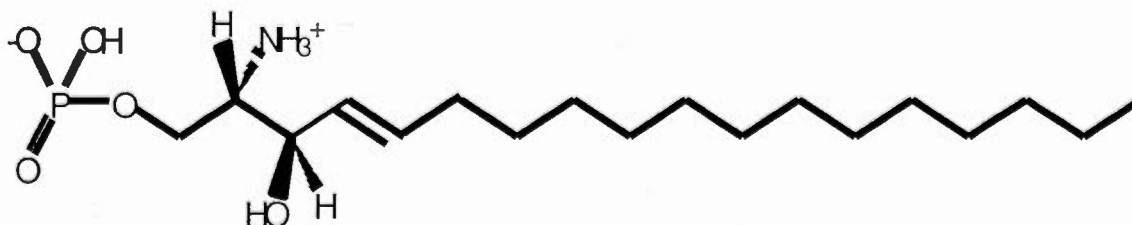
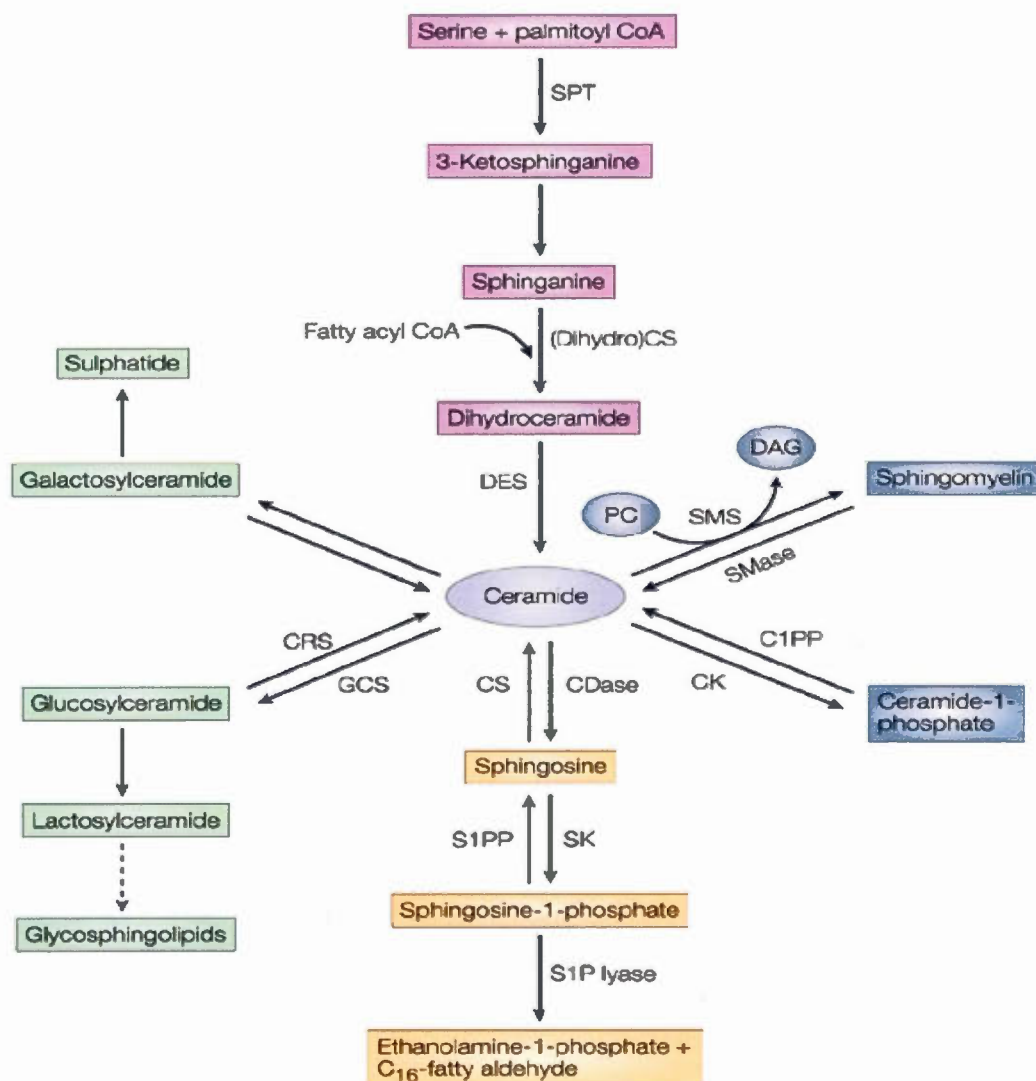


Figure 1.19 : Structure moléculaire de la sphingosine-1-phosphate.

1.7.2. Le métabolisme des sphingolipides

Dans les cellules, les céramides sont à la base de la synthèse de la S1P (Gulbins and Li 2006). Si les céramides sont impliqués dans le mécanisme d'apoptose (Hannun and Obeid 1995; Pettus et al. 2002), la S1P quant à elle joue un grand rôle dans les mécanismes de survie cellulaire (Radeff-Huang et al. 2004). Ceci suggère donc que la conversion des céramides en S1P pourrait représenter une façon de faire passer la cellule d'un état proapoptotique à un état prolifératif. Par conséquent, l'inhibition de la conversion des céramides en S1P pourrait permettre de stimuler la mort cellulaire au cours de maladies comme l'inflammation chronique ou le cancer.

Les céramides peuvent être synthétisés *de novo* ou par l'action de sphingomyélinases (Goni and Alonso 2002). L'activation de ces dernières a été observée suite à une stimulation des cellules par certains facteurs de croissance, certaines cytokines et par l'acide arachidonique (Marchesini and Hannun 2004). De plus, le stress cellulaire ainsi que le changement de potentiel redox de la cellule peuvent aussi activer les sphingomyélinases (Sawai and Hannun 1999). Suite à l'action des sphingomyélinases, les céramidases catalysent la déacétylation des céramides pour produire un acide gras libre et de la sphingosine. La sphingosine peut ensuite être phosphorylée par la sphingosine kinase pour donner de la S1P. La S1P pourra à son tour être clivée par la S1P lyase ou déphosphorylée par la S1P phosphatase (Ogretmen and Hannun 2004) (Figure 1.20).



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Figure 1.20 : Le métabolisme des sphingolipides (Ogretmen and Hannun 2004)

SPT : sérine palmitoylsynthase; **DihydroCS** : dihydrocéramide synthase; **DES** : désaturase; **SMS** : sphingomyéline synthase; **SMase** : sphingomyélinase; **GCS** : glucosylcéramide synthase; **CK** : céramide kinase; **C1PP** : céramide-1-phosphate phosphatase; **CS** : céramide synthase; **CDase** : céramidase; **SK** : sphingosine kinase; **S1PP** : sphingosine-1-phosphate phosphatase.

1.7.3. La sphingosine kinase

La sphingosine kinase (SphK) catalyse la phosphorylation de la sphingosine en S1P et, par conséquent, joue un double rôle, soit la synthèse de S1P et la diminution de sphingosine (Liu et al. 2002). En effet, l'augmentation de la concentration cellulaire en sphingosine est toxique pour la cellule d'où la nécessité de développer des mécanismes de contrôle (Maceyka et al. 2002). Deux différentes isoformes de la SphK ont été clonées, appelées SphK-1 et SphK-2 (Maceyka et al. 2005b) (Figure 1.21). Une large variété de stimuli peut moduler l'activité de la SphK et augmenter la production de S1P dans les cellules. Les facteurs de croissance, les agonistes des récepteurs liés aux petites protéines G, certaines cytokines, les esters de phorbol, certaines vitamines stimulent l'activité de la SphK (Pyne and Pyne 2002). La présence d'un site de liaison à la calmoduline (Sutherland et al. 2006), de sites de phosphorylation par les PKA, la caséine kinase II et les tyrosine kinases au sein de la séquence primaire de la SphK permettent de penser qu'il existe de nombreux mécanismes de régulation de cette enzyme (Pitson et al. 2000).

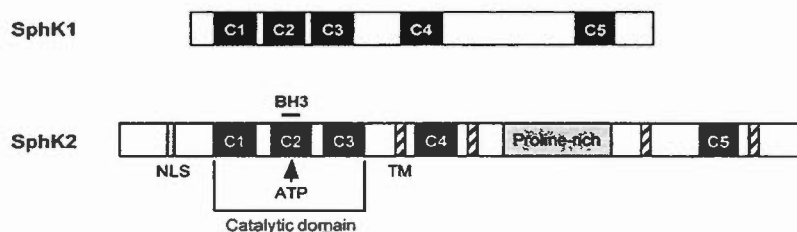
1.7.4. La sphingosine-1-phosphate lyase

La S1P lyase catalyse la dégradation de la S1P en aldéhyde palmitique et en phosphoéthanolamine (Van Veldhoven and Mannaerts 1991; Van Veldhoven et al. 2000). Cette enzyme est liée à la membrane et nécessite le phosphate de pyridoxal comme coenzyme (Van Veldhoven et al. 2000; Ikeda et al. 2004) (Figure 1.21). La S1P lyase a un pH optimum de 7.5 et est inhibée par les métaux lourds. Cette enzyme clive spécifiquement les isomères D-érythro des bases sphingoïdes phosphorylées, incluant la sphingosine-, la dihydrosphingosine- et la phytosphingosine-1-phosphate (Van Veldhoven et al. 2000). Cette enzyme est exprimée de façon ubiquitaire chez les mammifères, exception faite des plaquettes où la sphingosine lyase est totalement absente (Yatomi et al. 2000). Son expression est majoritaire dans le foie, les reins, les poumons, le cœur et le cerveau.

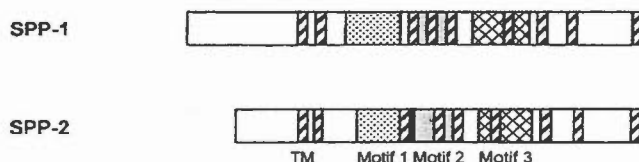
1.7.5. La sphingosine-1-phosphate phosphatase

Plusieurs lysophospholipides phosphatases (LPP1, 1a, 2 et 3) ont été clonées et présentent une spécificité de substrat relativement large puisqu'elle inclut la S1P, l'acide lysophosphatidique (LPA), l'acide phosphatidique et la céramide-1-phosphate (Pyne et al. 2005). La S1P est aussi spécifiquement déphosphorylée par la S1P phosphatase ou S1P phosphohydrolase (Mandala 2001). Deux isoformes de cette enzyme existent dans les cellules (Figure 1.21). Elles possèdent un domaine catalytique ayant une forte homologie avec le domaine catalytique des LPP. Cependant, la distance précise entre les différents domaines protéiques qui constituent le domaine catalytique permet d'établir la spécificité de l'enzyme envers la S1P (Mandala 2001).

SPHINGOSINE KINASES



S1P PHOSPHOHYDROLASES



S1P LYASE

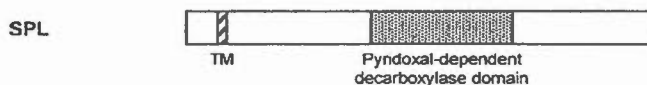


Figure 1.21 : Structure des sphingosine kinases, S1P phosphatases et S1P lyase

1.7.6. Activité de la S1P au niveau de l'endothélium vasculaire

De hauts niveaux de S1P se retrouvent dans le sang. En effet, les plaquettes, qui ne possèdent pas de S1P lyase, sont une grande source de S1P (Igarashi and Yatomi 1998). Les plaquettes libèrent la S1P en réponse à des stimuli prothrombotiques, tels que la thrombine, le collagène ou l'ADP (Yatomi et al. 2000). Grâce à cinq récepteurs transmembranaires (S1P-1 à S1P-5) couplés aux petites protéines G, anciennement appelés récepteurs EDG pour *Endothelial Differentiation Gene* (Hla 2001; Parrill et al. 2004), la S1P peut déclencher différentes voies de signalisation (MAPK, PI3K, Ca²⁺) qui stimulent la prolifération, la migration ou la différenciation cellulaire (Rosen and Goetzl 2005) (Figure 1.22). A cette stimulation directe des récepteurs à la S1P s'ajoutent des croisements de voies de signalisation entre les récepteurs à facteurs de croissance et les récepteurs à la S1P (Tanimoto et al. 2002; Igarashi et al. 2003). Outre la migration, la prolifération et la survie, la S1P stimule aussi le changement morphologique des cellules endothéliales (Argraves et al. 2004; Limaye et al. 2005; Kîmura et al. 2006) et la formation de structures de type capillaires sur Matrigel™ (Lee et al. 1999). Ces propriétés démontrent le rôle proangiogénique majeur de la S1P (Lee et al. 1999).

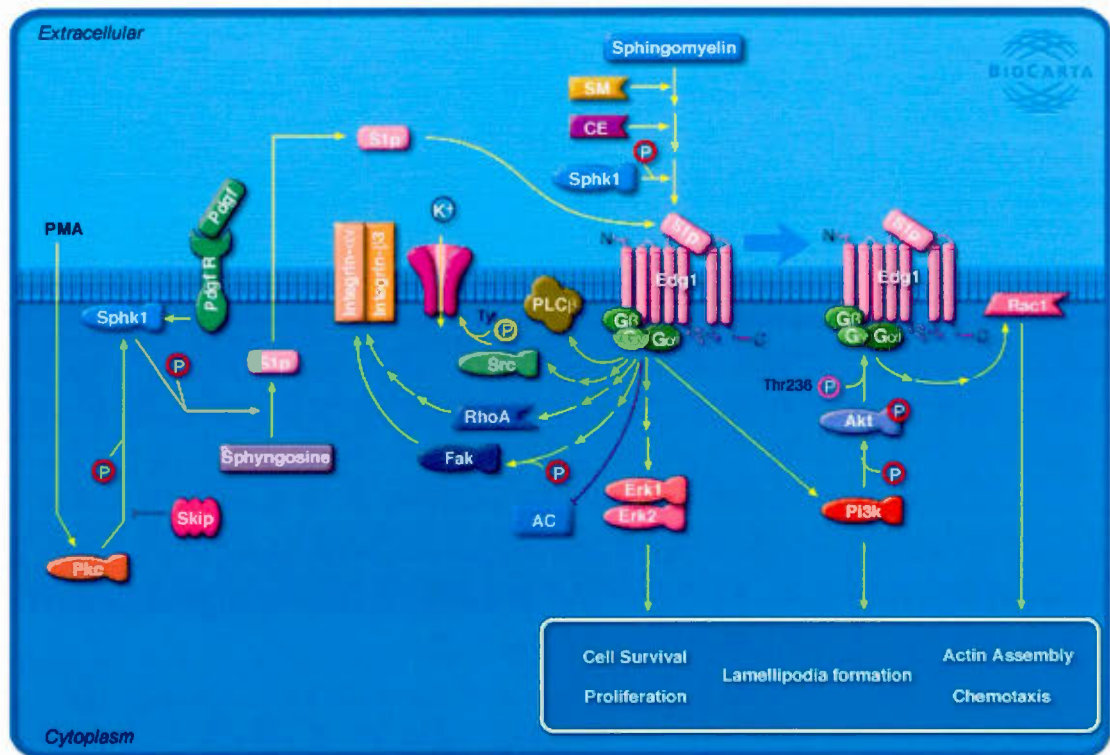


Figure 1.22 : Voies de signalisation activées par la S1P à la surface des cellules endothéliales.

La stimulation des récepteurs Edg à la surface des cellules active différentes voies de signalisation telles que la voie PI3K/Akt ou la voie des MAPKinases. La signalisation par ces voies entraîne diverses réponses cellulaires telles que la migration, la prolifération ou la survie cellulaire.

1.7.7. S1P et cancer

De nombreuses données mettent en évidence le rôle de la S1P dans la progression tumorale, notamment dans le développement des tumeurs gliales (Van Brocklyn et al. 2003; Van Brocklyn et al. 2005). Non seulement la S1P stimule la prolifération et le potentiel métastatique de cellules de glioblastomes, mais on retrouve aussi ses récepteurs en abondance dans la plupart de ces tumeurs (Van Brocklyn et al. 2002). De plus, des concentrations importantes de S1P ont été mesurées dans le cerveau (Edsall and Spiegel 1999). Cependant, le rôle de la S1P dans le développement tumoral pourrait être spécifique de l'origine tissulaire de la tumeur. En effet, sur des cellules de cancer du sein, la S1P inhibe le phénotype tumoral (Wang et al. 1999). Cet effet serait dû à l'activité intracellulaire de la S1P, plutôt qu'à l'activation des récepteurs membranaires, peu exprimés dans les cellules mammaires étudiées. Ceci suggère donc que l'activité protumorale de la S1P dépend du taux d'expression de ses récepteurs, mais elle est aussi très probablement conditionnée par les niveaux cellulaires de S1P phosphatase et de sphingosine kinase qui interconvertissent S1P et sphingosine (Pyne 2002).

Une dernière caractéristique de la S1P pouvant jouer un rôle important dans le développement tumoral est son pouvoir angiogénique (Panetti 2002). En effet, l'inactivation du gène codant pour le récepteur S1P-1 a démontré que la S1P est un facteur essentiel du développement vasculaire embryonnaire mais aussi de l'angiogenèse cérébrale, ce qui suggère qu'elle participe tant à des phases d'angiogenèse précoces que tardives (Liu et al. 2000; Chae et al. 2004; Krump-Konvalinkova et al. 2005). Ainsi, la S1P pourrait non seulement protéger les cellules tumorales en activant leurs voies de signalisation antiapoptotiques (Pyne 2002), mais également faciliter la vascularisation des tumeurs, une étape-clé de leur croissance. On peut donc imaginer que d'éventuels inhibiteurs de la sphingosine kinase (French et al. 2006), de la S1P (Visentin et al. 2006) ou des antagonistes des récepteurs à la

S1P (LaMontagne et al. 2006) pourraient être particulièrement actifs sur certaines tumeurs en les attaquant sur deux fronts distincts.

1.8. LES THÉRAPIES ANTIANGIOGÉNIQUES

L'utilisation de molécules angiostatiques pour le traitement du cancer est une voie prometteuse et présente, par rapport aux thérapies conventionnelles, certains avantages (Cao 2005; Ferrara and Kerbel 2005) (Figure 1.23). D'abord, elle fait abstraction de la très grande variété phénotypique et génotypique qui caractérise les cellules tumorales : ces molécules ciblent les cellules endothéliales engagées dans la formation des néovaisseaux tumoraux qui favorisent la propagation de la plupart des cancers. Ensuite, le risque de résistance aux agents angiostatiques semble, a priori, beaucoup plus faible que celui associé aux agents pharmacologiques (chimiothérapie) même si de récentes études cliniques laissent à penser que les cellules tumorales pourraient tout de même s'adapter aux thérapies antiangiogéniques (van Kempen and Leenders 2006). Enfin, la réduction de la vascularisation tumorale diminue les risques de dissémination métastatique.

L'inhibition des métalloprotéases de matrice, de la transduction des signaux, le blocage des récepteurs membranaires, l'inhibition des fonctions cellulaires endothéliales (migration, prolifération, tubulogénèse), le blocage des activateurs de l'angiogénèse et la destruction du lit vasculaire sont autant de stratégies permettant de bloquer le développement de la vascularisation tumorale (Siemann et al. 2000) (Figure 1.24). L'un des défis des recherches actuelles est donc d'élaborer des outils pharmacologiques capables de bloquer les cibles thérapeutiques définies précédemment. Étant donné que des corrélations cliniques ont été établies entre le pouvoir invasif et métastasant d'un certain nombre de cancers et la surproduction de VEGF (Figure 1.9 et 1.10) et de FGF-2 (Garcea et al. 2005; Kumar and Kratzke 2005; Yasui et al. 2005), une stratégie visant à inactiver ces facteurs angiogéniques *in vivo* avec des anticorps neutralisants ou en inhibant la réponse cellulaire a donné des résultats particulièrement encourageants (Kerr 2004). L'autre possibilité est d'utiliser des facteurs angiostatiques. Dans cette perspective, une grande attention a été portée

sur l'angiostatine et sur l'endostatine étant donné leur spécificité d'action pour l'endothélium vasculaire (Wahl et al. 2004; Huszthy et al. 2006; Passey 2006). Un grand nombre de ces composés sont entrés en phase clinique (Tableau 1.5). Le blocage de la fonction des intégrines, soit par l'utilisation d'anticorps monoclonaux spécifiques (Varner et al. 1999; Trikha et al. 2004), soit par l'utilisation de molécules naturelles liant spécifiquement les intégrines (Golubkov et al. 2003; Marcinkiewicz et al. 2003) donnent aussi des résultats prometteurs. En dehors de la thérapeutique médicamenteuse antiangiogénique, la thérapie génique, permettant d'exprimer localement et spécifiquement certaines molécules antiangiogéniques, semble aussi donner des résultats encourageants (Passey 2006). Enfin, le développement de l'immunothérapie antiangiogénique permet aussi d'augmenter l'arsenal thérapeutique de lutte contre le cancer (Hou et al. 2004a; Cardones and Banez 2006; Okaji et al. 2006).

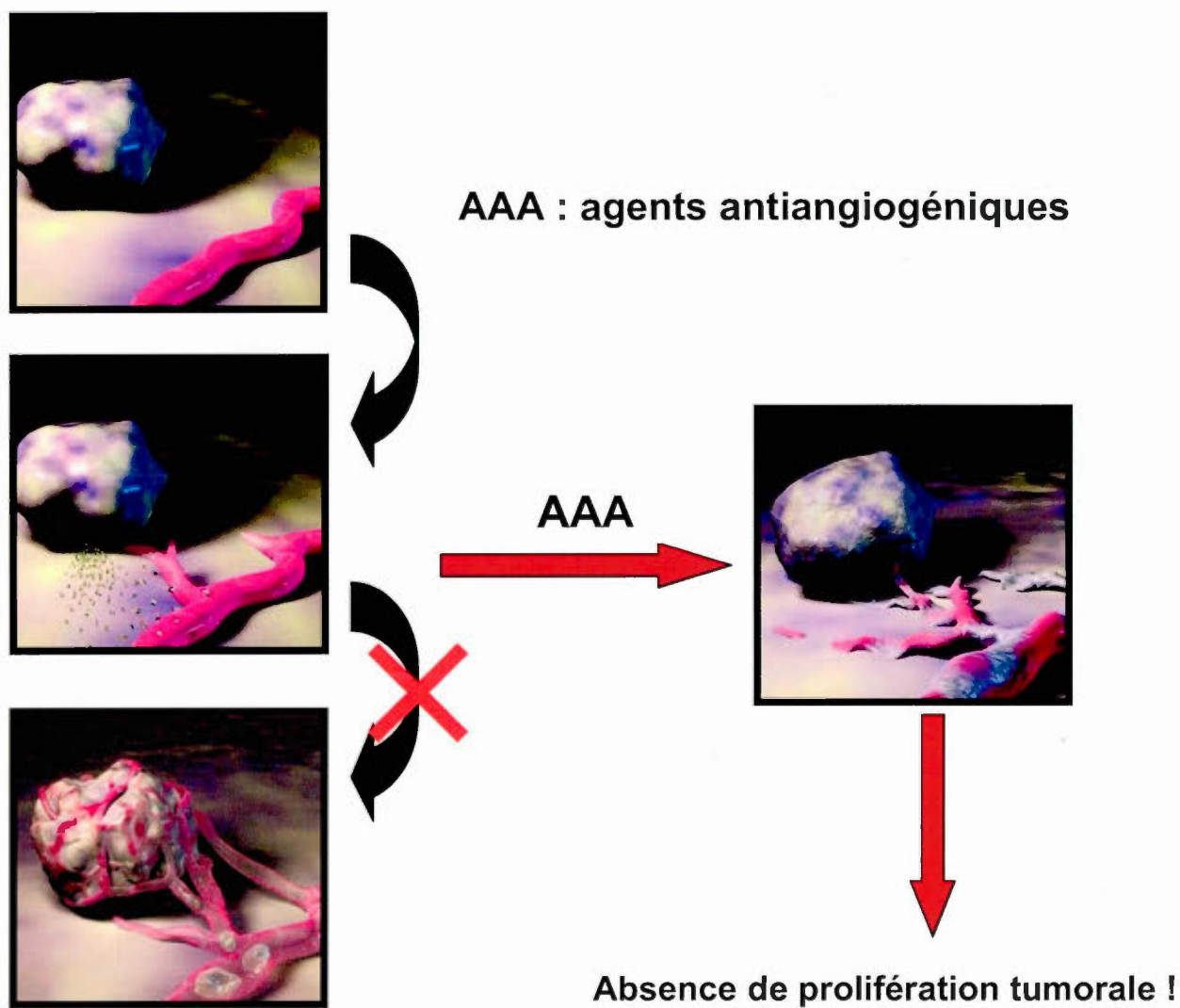


Figure 1.23 : Principe des thérapies antiangiogéniques.

Les agents antiangiogéniques permettent de bloquer la croissance des vaisseaux sanguins menant au développement tumoral. L'inhibition de la croissance des vaisseaux bloque le développement tumoral et empêche la dissémination métastatique des cellules cancéreuses.

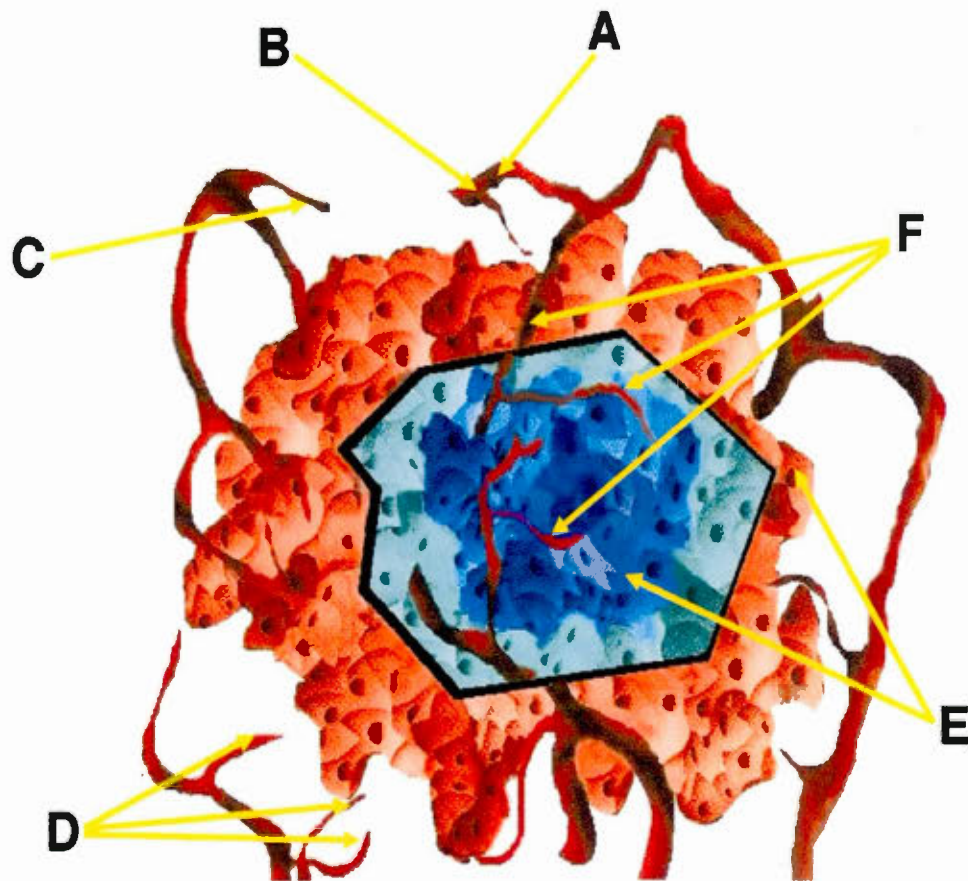


Figure 1.24 : Les stratégies des thérapies antiangiogéniques.
(Siemann et al. 2000).

A : Inhibition des MMPs, **B** : Inhibition de la transduction des signaux, **C** : Blocage des récepteurs membranaires, **D** : Inhibition des fonctions cellulaires endothéliales (migration, prolifération, tubulogénèse), **E** : blocage des activateurs de l'angiogénèse, **F** : destruction du lit vasculaire existant.

Médicament	Tumeur	Mode d'action
Phase I		
Combretastatin A4	Cancers avancés	Destruction du cyto-squelette des cellules endothéliales et induction d'un collapsus vasculaire
PTK787/ZK2284	Cancers avancés	Bloque le signal VEGF-récepteur des cellules endothéliales
Endostatin	Cancers avancés	Inconnu
BMS-275291	Cancers avancés	Inhibiteur synthétique des métallo-protéinases, inhibe la dégradation de la matrice extracellulaire
SU6668	Cancers avancés	Bloque le signal du facteur de croissance des cellules endothéliales
Phase II		
EMD121974	Gliome	Antagoniste de l'intégrine vasculaire $\alpha V\beta 3$
Vitaxin		Anti- $\alpha V\beta 3$ mAb humanisé qui inhibe l'adhésion des cellules endothéliales
CGS-27023A		Inhibiteur synthétique des métallo-protéinases, inhibe la dégradation de la matrice extracellulaire
TNP-470		Inhibe la prolifération endothéliale
CAI	Ovalres, reins	Inhibe la prolifération endothéliale
Interleukine-12	Sarcome de Kaposi	Inhibe la prolifération endothéliale en induisant l'Interféron- α
IM862		Inconnu
Anti-VEGF Ab		Bloque la prolifération endothéliale
Phase III		
COL-3	Cerveau, sarcome de Kaposi	Inhibiteur synthétique des métallo-protéinases, inhibe la dégradation de la matrice extracellulaire
SU5416	Côlon, rectum	Bloque le signal du récepteur VEGF
Thalidomide	Beaucoup	Inconnu
AG3340		Inhibiteur synthétique des métallo-protéinases, inhibe la dégradation de la matrice extracellulaire
Neovastat	Poumons, reins	Inhibiteur naturel des métallo-protéinases, inhibe la dégradation de la matrice extra-cellulaire
Interferon- α	Beaucoup	Inhibe la prolifération endothéliale
IM862		Mécanisme inconnu
BMS-255291	Poumons	Inhibiteur synthétique des métallo-protéinases, inhibe la dégradation de la matrice extracellulaire
Marimastat	Poumons, seins	Inhibiteur synthétique des métallo-protéinases, inhibe la dégradation de la matrice extracellulaire

Tableau 1.6 : Quelques molécules en essais cliniques en 2003
(d'après les données du *National Cancer Institute*)

1.9. MOLÉCULES NATURELLES ET TRAITEMENT DU CANCER

1.9.1. Les nutraceutiques

1.9.1.1. Définition

Selon l'Office Québécois de la Langue Française, le terme «nutraceutique» fait référence à l'ingrédient actif, présent à l'état naturel dans un aliment, qui procure un effet bénéfique pour la santé.

1.9.1.2. La nutrathérapie

La nutrathérapie peut être comparée à une chimiothérapie utilisant l'ensemble des molécules anticancéreuses présentes dans les aliments pour combattre les cellules cancéreuses qui se développent spontanément ou les cellules endothéliales qui vascularisent les tumeurs. La consommation régulière de fruits et légumes correspond donc à une chimiothérapie préventive non toxique.

1.9.2. Un nutraceutique avéré, le thé vert.

1.9.2.1. Généralités

Après l'eau, le thé est la boisson la plus consommée dans le monde. Il s'en boit 25 000 tasses par seconde. Le thé est principalement cultivé en Asie, en Afrique et dans certains pays d'Amérique latine et d'Europe de l'Est. Il n'existe qu'une espèce de théier, le *Camellia sinensis*, qui serait originaire du sud-ouest de la Chine, et deux grandes variétés : la variété *sinensis* de Chine et la variété *assamica* de l'Inde. Aujourd'hui, la Chine est le deuxième pays producteur de thé. Suivent deux anciennes colonies anglaises, le Sri Lanka et le Kenya, qui ont amorcé plus tard la production de thé.

1.9.2.2. Les catéchines du thé vert

Plusieurs études suggèrent qu'une prise quotidienne de thé vert réduit les risques de développer divers types de cancer dont ceux de l'estomac, du côlon, de la prostate et du sein (Cooper et al. 2005). Une simple tasse de thé est un mélange complexe de plus de 500 substances actives. Outre les différences liées à la nature du thé et à la durée d'infusion, la nature et la température de l'eau entraînent aussi une variabilité extrême de la composition de la boisson. Les principaux composants du thé sont, par ordre d'importance, l'eau, les tanins, les protéines, les glucides, les acides organiques, la caféine, les vitamines, les minéraux et des centaines de substances aromatiques (Bokuchava and Skobeleva 1980).

Les analyses biochimiques des constituants du thé vert ont montré qu'un tiers de la matière sèche des feuilles de thé est constitué de flavonoïdes. Ces polyphénols, aussi appelés catéchines, sont les principaux éléments actifs responsables des propriétés anti-cancéreuses du thé (Liao et al. 2001). Les principales catéchines sont l' (-)-épicatéchine (EC), l' (-)-épicatéchine-3-gallate (ECG), l' (-)-épigallocatechine (EGC) et l' (-)-épigallocatechine-3-gallate (EGCG) (Figure 1.25). La majorité des effets biologiques du thé vert sont attribués à l'EGCG (Fujiki 2005). Cependant, le contenu en catéchine des différentes feuilles de thé varie selon le degré de fermentation et, par conséquent, les thés qui en contiennent le plus sont, par ordre décroissant, les thés blancs, les thés verts, les thés oolongs et les thés noirs, ces derniers en contenant très peu (Figure 1.26).

La structure particulière de ces polyphénols semble être à l'origine de leurs activités cellulaires. D'abord, la structure chimique des catéchines leur donne un fort pouvoir antioxydant, leur permettant d'inactiver les radicaux libres et de chélater certains ions comme le Ca^{2+} ou le Mg^{2+} . De plus, les polyphénols peuvent aussi subir une autooxydation en solution (Hou et al. 2004b; Hou et al. 2005). Ces deux propriétés sont importantes pour les activités biologiques des polyphénols.

Cependant, la structure polyphénolique des catéchines leur permet aussi d'interagir directement avec certaines protéines comme, par exemple, les protéines salivaires riches en proline (Jobstl et al. 2004), la fibronectine, le fibrinogène, certaines protéines du plasma, les protéines riches en histidine (Sazuka et al. 1996) et le récepteur à la laminine de 67 kDa (Tachibana et al. 2004). Leur pouvoir antioxydant et leur capacité à interagir avec les protéines cellulaires représentent les bases physicochimiques de l'activité biologique des polyphénols.

Plusieurs études épidémiologiques ont déjà suggéré que les gens buvant du thé vert développaient moins de cancers du tractus gastro-intestinal (Arab and Il'yasova 2003; Borrelli et al. 2004). On sait également que les polyphénols du thé inhibent la formation et la croissance de tumeurs chez les rats et les souris (Das et al. 2002; Kemberling et al. 2003; Yang et al. 2005a). Le thé vert, et plus particulièrement l'EGCG, possède aussi des propriétés antiangiogénique remarquables (Demeule et al. 2002b). L'EGCG agit à divers niveaux : il bloque l'activation du récepteur au VEGF à la surface des cellules endothéliales (Lamy et al. 2002), inhibe l'activité des métalloprotéases matricielles qui facilitent la progression tumorale (Demeule et al. 2000) et affecte la transcription de certains gènes (Chen and Zhang 2003). D'autres cibles moléculaires de l'EGCG restent encore à être caractérisées.

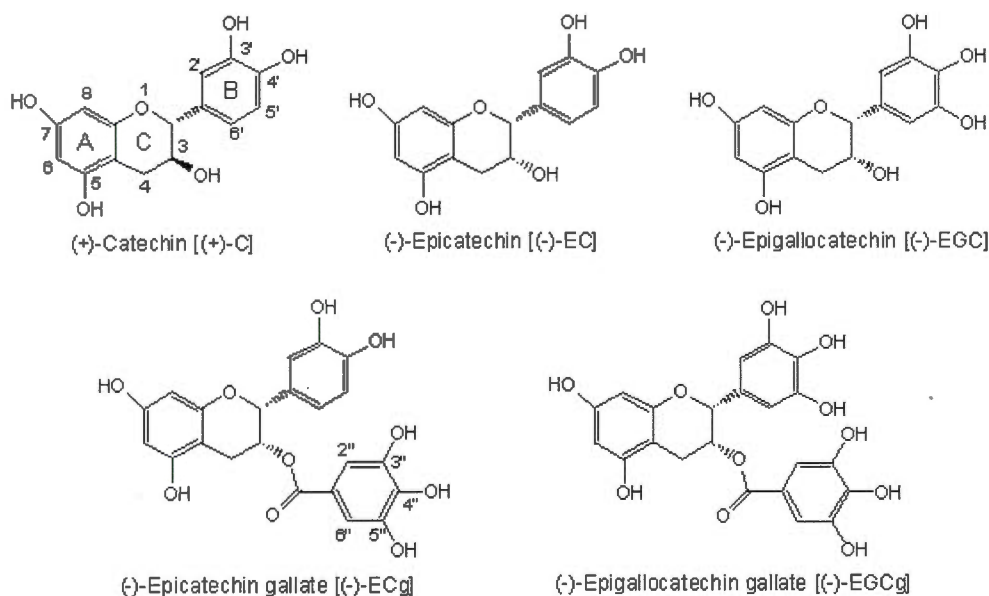


Figure 1.25 : Structure des principales catéchines du thé vert

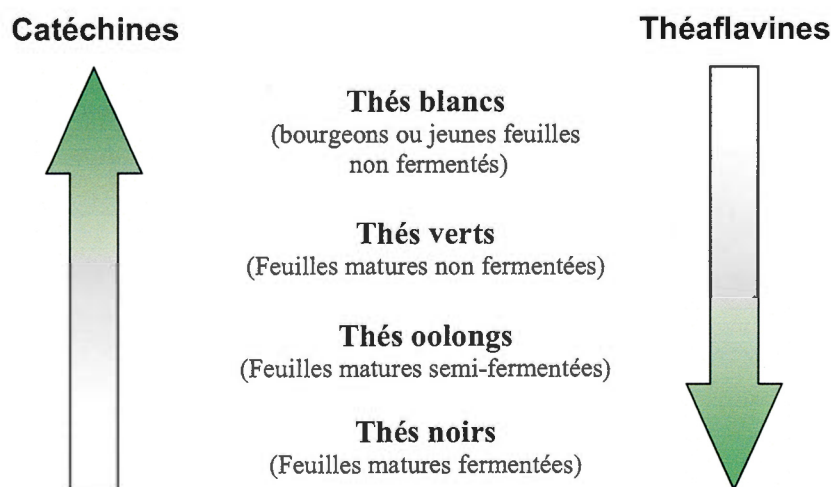


Figure 1.26 : Relation entre les différents types de thé et leur concentration en polyphénols.

1.9.3. Les venins de serpents

1.9.3.1. Généralités

Les venins de serpent sont des mélanges complexes de polypeptides et d'enzymes hydrolytiques (protéases, hémorragines, enzymes coagulo-actifs, kininogénases, phospholipases), qui peuvent provoquer une série de dommages biologiques (cytotoxicité locale, neurotoxicité, hématotoxicité, myotoxicité) (Bazaa et al. 2005). Les protéines constituent 90 à 95 % du poids sec des venins et sont responsables de la quasi totalité des effets biologiques de ces derniers. Parmi les centaines, voire les milliers de protéines différentes contenues dans un venin de serpent, on trouve des toxines, en particulier des neurotoxines, cardiotoxines, hémotoxines, mais aussi des protéines non toxiques pouvant posséder certaines propriétés pharmacologiques, et de nombreuses enzymes.

1.9.3.2. Venins et cancer

Les milliers de molécules présentes dans les venins de serpents représentent des armes mortelles d'une efficacité remarquable. Cependant, isolées, elles peuvent devenir des armes redoutables pour lutter contre le cancer (Pal et al. 2002). En effet, les venins de serpent contiennent des composants uniques qui peuvent affecter les interactions entre les cellules et la MEC et ainsi bloquer le développement tumoral. Deux grandes classes de peptides issus de venins de serpents ont été caractérisée pour leurs propriétés antitumorales : les désintégrines et les lectines de type C.

1.9.3.2.1. Les désintégrines

Les désintégrines sont des peptides de faible poids moléculaire, riches en cystéine, isolés principalement des venins de serpents (Calvete et al. 2005; Marcinkiewicz 2005). Les désintégrines contiennent un motif RGD leur permettant

d'interagir avec toutes les intégrines de type RGD en tant qu'antagonistes de l'adhérence (Gould et al. 1990). Elles lient principalement les intégrines $\alpha\text{IIb}\beta 3$, $\alpha\text{I}\beta 1$, $\alpha 6\beta 4$, $\alpha 5\beta 1$ et $\alpha\text{V}\beta 3$ exprimées à la surface des plaquettes, des cellules endothéliales et de certaines cellules tumorales (Pfaff et al. 1994). Ainsi, les désintégrines permettent d'inhiber l'agrégation plaquettaire, l'adhérence et la migration cellulaire et par conséquent, l'angiogenèse et le développement des tumeurs (Olfa et al. 2005; Pyrko et al. 2005). Certaines désintégrines peuvent aussi lier la glycoprotéine GPIb à la surface des plaquettes, bloquant dans le même temps, l'interaction de cette protéine avec le facteur de Von Willebrand menant à l'activation plaquettaire et inhibant l'agrégation plaquettaire (Peng et al. 1993). Par conséquent, les désintégrines possèdent des propriétés antiangiogéniques, antitumorales et antithrombotiques.

1.9.3.2.2. Les lectines de type C

Les lectines sont des protéines non enzymatiques qui reconnaissent et fixent spécifiquement les oses ou dérivés d'oses, constituants habituels des oligosaccharides des glycoprotéines (Dodd and Drickamer 2001; Kilpatrick 2002). On les trouve en grande quantité dans le règne végétal mais aussi chez les bactéries, les œufs de poisson, les escargots, les vertébrés et les champignons. Les lectines sont regroupées en deux groupes, les lectines de type S ou galectines (Fred Brewer 2002) et les lectines de type C (Zelensky and Gready 2005), selon que leur liaison aux oses requiert (type C) ou non (type S) la présence de cations divalents.

Des peptides de type lectines de type C (*C-type lectins like proteins* ou *CLPs*) ont aussi été isolés à partir de venins de serpents appartenant aux familles des *Elapidae*, des *Viperidae* et des *Crotalidae* (Morita 2005). Bien qu'ayant un nom similaire aux lectines de type C classiques, les CLPs diffèrent de ces dernières par l'absence d'activité lectine. Leur dénomination provient du fait que leur séquence primaire en acides aminés possède 15 à 40% d'homologie avec la séquence en acides

aminés du domaine de liaison aux oses des lectines de type C classiques (Morita 2005). Les CLPs sont des protéines de 30kDa formées de deux sous-unités liées par un pont disulfure (Morita 2005; Zelensky and Gready 2005). En dépit de leur fort taux d'homologie (entre 40 et 70%), les différentes CLPs connues présentent des propriétés biologiques très diverses (Sarray et al. 2001; Morita 2005). En effet, certaines CLPs empêchent la formation du complexe facteur de Von Willebrand-glycoprotéine GPIb, bloquant l'agrégation plaquettaire tandis que d'autres activent la formation de ce complexe, menant à l'agrégation plaquettaire (Andrews and Berndt 2000). En plus de ces activités pro- ou antithrombotiques, les CLPs modulent aussi les propriétés d'adhérence des intégrines, affectant le développement tumoral et l'angiogenèse (Du et al. 2001; Chung et al. 2004; Sarray et al. 2004). Par conséquent, à l'instar des désintégrines, les CLPs représentent de nouveaux espoirs thérapeutiques pour le traitement des tumeurs.

1.10. PRÉSENTATION DU PROJET DE RECHERCHE

Les travaux de la présente thèse ont eu pour objectif de mettre en évidence de nouvelles voies possibles pour le traitement des tumeurs cérébrales. La première partie de notre étude, comprenant les deux premiers articles, a eu pour but de caractériser de nouvelles molécules d'origine naturelle pour leur pouvoir antitumoral et/ou antiangiogéniques. Nous avons, à dessein, restreint notre étude à des molécules affectant les intégrines. La seconde partie de cette thèse a eu pour but de mieux caractériser le rôle de la S1P au niveau de l'endothélium cérébral. Les résultats exposés dans cette thèse permettront de mieux comprendre le fonctionnement de l'endothélium cérébral et d'améliorer les traitements existants pour les tumeurs cérébrales.

1.10.1. Régulation des propriétés invasives des médulloblastomes par l'EGCG.

Les médulloblastomes sont les tumeurs cérébrales pédiatriques les plus fréquentes. Elles représentent 20% de toutes les tumeurs cérébrales. Les traitements actuels ont des effets secondaires importants sur le système nerveux central des patients (Packer et al. 1999; Rood et al. 2004). Par conséquent, il est important de trouver de nouvelles méthodes thérapeutiques. Les polyphénols du thé vert présentent des propriétés antitumorales importantes, d'où l'intérêt de leur utilisation potentielle pour le traitement des médulloblastomes (Zaveri 2006). Puisque les intégrines sont impliquées dans les mécanismes d'adhérence et de migration des cellules tumorales, il était intéressant de savoir si ces protéines pouvaient aussi être des cibles de l'action antitumorale des polyphénols du thé vert.

Le but de cette étude a été de caractériser les effets antitumoraux de l'EGCG, le principal polyphénol du thé vert, sur les cellules DAOY de médulloblastome. Nous avons déterminé l'expression d'un certain nombre d'intégrines à la surface de ces

cellules. Puis, nous avons étudié l'effet de l'EGCG sur les propriétés d'adhérence et de migration des cellules DAOY sur différentes protéines purifiées de la matrice extracellulaire. Enfin, nous avons déterminé les modulations de l'expression des intégrines suite au traitement avec l'EGCG. Nous avons pour cela utilisé la cytométrie de flux et la RT-PCR afin de déterminer ces variations tant au niveau transcriptionnel que traductionnel. Nous avons déterminé que l'expression de l'intégrine $\beta 1$ est augmentée en réponse à l'EGCG, tant au niveau transcriptionnel que traductionnel. Cette étude a permis de démontrer que l'EGCG pouvait inhiber le caractère invasif des médulloblastomes en modifiant l'expression des intégrines.

1.10.2. Propriété antiangiogénique de la lébectine, une lectine de type C, isolée du venin de *Macrovipera lebetina*.

L'angiogenèse cérébrale joue un rôle fondamental dans le développement des tumeurs cérébrales. Par conséquent, cibler la vascularisation de ces tumeurs permet d'attaquer la tumeur sur un second front, sans créer d'effets secondaires (Kieran 2004). Les intégrines exprimées à la surface des cellules endothéliales sont nécessaires à la migration de ces cellules vers la source du stimulus angiogénique, autrement dit la tumeur. Par conséquent, bloquer les fonctions des intégrines endothéliales revient à immobiliser les cellules endothéliales. Les intégrines vasculaires sont principalement les intégrines $\alpha V\beta 3$, $\alpha V\beta 5$ et $\alpha 5\beta 1$. Depuis quelques années ont été mis en évidence des peptides, isolés des venins de plusieurs serpents, dont les propriétés anti-intégrines sont remarquables (Marcinkiewicz 2005). De plus, il semble qu'ils interagissent spécifiquement avec certaines intégrines comme les intégrines vasculaires. Par conséquent, leur utilisation en thérapie antiangiogénique représente un intérêt grandissant du point de vue clinique.

Nous avons focalisé notre étude sur la mise en évidence des propriétés antiangiogéniques de la lébectine, une lectine de type C isolée du venin de la vipère

Macrivipera lebetina (Sarray et al. 2004). Nous avons mesuré l'effet de la lébectine sur les propriétés de migration, d'adhérence, de tubulogénèse et de prolifération des cellules endothéliales cérébrales humaines HBMEC. Nous avons aussi mesuré les effets de la lébectine sur l'angiogénèse *in vivo*, en utilisant les essais sur membranes chorioallatoidiennes de poulet et en implant de Matrigel™ chez la souris. Enfin, nous avons mis en évidence les interactions entre le lébectine et certaines intégrines vasculaires. Cette étude a permis de caractériser la lébectine en tant que nouvel agent antiangiogénique pour le traitement des tumeurs cérébrales.

1.10.3. Rôle de la sphingosine-1-phosphate au cours du processus angiogénique cérébral.

L'angiogénèse cérébrale est un processus régulé par divers facteurs solubles, tels que des facteurs de croissance, des cytokines ou encore certains lipides bioactifs comme la sphingosine-1-phosphate (S1P). La S1P a été caractérisée comme un facteur clairement proangiogénique dans de nombreux types cellulaires (Panetti 2002). Cependant, son rôle au niveau de l'endothélium vasculaire cérébral est encore inconnu. Plus précisément, aucune étude n'a permis de mettre en évidence le rôle de la S1P au cours du processus angiogénique cérébral.

Dans notre troisième étude, nous avons mesuré l'effet de la S1P sur les propriétés migratoires et sur la tubulogénèse des cellules endothéliales cérébrales de rat RBE4. Nous avons effectué des essais de migration *in vitro* en chambre de Boyden modifiée et de tubulogénèse sur Matrigel™, en présence ou non de S1P. Bien que la S1P stimule fortement l'angiogénèse périphérique, nous avons mis en évidence les propriétés antiangiogéniques de la S1P sur l'endothélium cérébral. De plus, nous avons déterminé que l'absence de MT1-MMP, une métalloprotéase de matrice

membranaire, dans les cellules RBE4 pouvait expliquer ces observations. Cette étude est la première à avoir démontré un rôle antiangiogénique de la S1P.

1.10.4. Régulation des fonctions de la glycoprotéine P par la sphingosine kinase.

La glycoprotéine P (P-gp) participe à la protection du cerveau en expulsant les xénobiotiques qui y pénètrent. Cependant, elle joue aussi un rôle important dans la résistance des tumeurs aux médicaments. Par conséquent, une meilleure compréhension des mécanismes de régulation de cette protéine permettrait de rendre les traitements anticancéreux plus efficaces. La sphingosine kinase (SphK) est une protéine dont la surexpression dans les tumeurs cérébrales est corrélée avec un faible pronostic de survie (Van Brocklyn et al. 2005). De plus, son expression augmente dans de nombreuses cellules chimiorésistantes. La P-gp étant fortement exprimée au niveau de l'endothélium cérébral, il serait intéressant d'examiner l'expression de la SphK au niveau de l'endothélium cérébral et de mesurer son effet sur l'expression et la fonction de la P-gp.

Dans un premier temps, nous avons mesuré l'expression de la SphK dans des cellules endothéliales isolées de cerveaux sains ou de glioblastomes implantés chez le rat. Ensuite, nous avons étudié l'effet de la surexpression de la SphK sur les fonctions de la P-gp, dans les cellules RBE4. Enfin, nous avons mesuré l'impact de la S1P sur la fonction de transport de la P-gp. Cette étude est la première à mettre en évidence l'implication de la SphK dans la régulation des fonctions de la P-gp. De plus, elle a permis de mettre en évidence que l'expression et/ou l'activité de la SphK est une cible intéressante pour les traitements antiangiogéniques et antitumoraux au niveau cérébral.

CHAPITRE II

CONTRIBUTIONS PERSONNELLES

Article 1: Medulloblastoma cell invasion is inhibited by green tea (-) epigallocatechin-3-gallate

Anthony Pilorget^{1,2}, Virginie Berthet², José Luis², Albert Moghrabi³, Borhane Annabi¹ and Richard Béliveau^{1,3}

¹Laboratoire de Médecine Moléculaire and ³Division of Hematology-Oncology, Centre de Cancérologie Charles Bruneau, Hôpital Sainte-Justine and Université du Québec à Montréal, Montreal, Quebec, Canada, and ²UMR CNRS 6032 Faculté de Pharmacie, 27, bd Jean Moulin, 13385 Marseille, Cedex 5, France

Correspondence : Richard Béliveau, PhD, Laboratoire de Médecine Moléculaire, Centre de Cancérologie Charles Bruneau, Hôpital Sainte-Justine, 3175 Côte Sainte-Catherine, Montréal, Québec, Canada, H3T 1C5, Tel: (514) 987-3000 ext. 8551, Fax: (514) 987-0246, E-mail: oncomol@nobel.si.uqam.ca

Running title: EGCG regulates β 1 integrin in medulloblastoma

Key words: cell adhesion, migration, integrin, EGCG, green tea, medulloblastoma

Abbreviations: EGCG, (-) epigallocatechin-3-gallate; EGC, (-) epigallocatechin; ECM, extracellular matrix; MMP, matrix metalloproteinases

CONTRIBUTION

Ce premier article a eu pour but de caractériser l'effet de l'EGCG, un polyphénol extrait du thé vert, sur l'expression des intégrines dans les cellules de médulloblastome DAOY. Il fait suite aux nombreuses études démontrant les propriétés antitumorales des polyphénols du thé vert. Cet article est le premier à avoir mis en évidence le rôle de l'EGCG dans la régulation de l'expression des intégrines.

J'ai réalisé les expériences, en collaboration avec le Dr Virginie Berthet, sous la supervision directe du Dr Borhane Annabi et de mes deux co-directeurs, le Dr Richard Béliveau et le Pr José Luis. J'ai rédigé cet article avec l'aide précieuse du Dr Borhane Annabi. La relecture de cet article a été faite par le Pr José Luis, le Dr Richard Béliveau et le Dr Denis Gingras. Cet article a été publié dans la revue scientifique *Journal of Cellular Biochemistry* en novembre 2003.

RÉSUMÉ EN FRANÇAIS

L'épigallocatechine gallate (EGCG), le polyphénol le plus présent dans le thé vert, peut se retrouver dans le cerveau après l'ingestion d'une tasse de thé. Possédant certaines propriétés antitumorales, il pourrait donc influencer sur le développement des tumeurs cérébrales. Puisque les intégrines, en permettant aux cellules cancéreuses d'adhérer à la matrice extracellulaire et de migrer, jouent un rôle majeur au cours du développement tumoral, nous avons étudié les effets de l'EGCG sur l'expression de diverses intégrines dans les cellules de médulloblastome DAOY. Par une analyse en cytométrie de flux, nous avons déterminé l'expression de certaines intégrines à la surface des cellules DAOY. On observe une forte expression des intégrines $\alpha 2$, $\alpha 3$ et $\beta 1$, de même que des intégrines αV et $\beta 3$. Nous démontrons que l'EGCG inhibe la migration des cellules DAOY sur collagène en augmentant leur adhérence sur ce substrat *via* l'augmentation de l'expression de l'intégrine $\beta 1$, tant au niveau transcriptionnel que traductionnel. Nos résultats suggèrent donc que l'EGCG pourrait être utilisé en tant qu'agent nutraceutique en inhibant le caractère invasif des médulloblastomes.

ABSTRACT

Epigallocatechin-3-gallate (EGCG), the major green tea polyphenol, can reach the brain following oral intake and could thus act as an antitumoral agent targeting several key steps of brain cancer cells invasive activity. Because integrin-mediated extracellular matrix recognition is crucial during the cell adhesion processes involved in carcinogenesis, we have investigated the effects of EGCG on different cellular integrins of the pediatric brain tumor-derived medulloblastoma cell line DAOY. Using flow cytometry, we report the levels of expression of several cell surface integrins in DAOY. These include high expression of $\alpha 2$, $\alpha 3$ and $\beta 1$ integrins, as well as αv and $\beta 3$ integrins. Moreover, we provide evidence that EGCG can antagonize DAOY cell migration specifically on collagen by increasing cell adhesive ability through specific gene and protein upregulation of the $\beta 1$ integrin subunit. Our results suggest that this naturally occurring green tea polyphenol may thus be used as a nutraceutical therapeutic agent in targeting the invasive character of medulloblastomas.

INTRODUCTION

Brain tumors are the second leading cause of cancer in children. Among these tumors, the highly metastatic medulloblastomas represent approximately 25% of all pediatric intracranial neoplasms [Saran, 2002]. These neoplasms arise from the cerebellum and mainly affect children between ages 5 to 15 [Weil, 2002]. Medulloblastomas are currently treated by surgical intervention, generally combined with aggressive chemotherapy [Mazzola and Pollack, 2003; Kaaijk et al., 2003]. However, the 6-year survival rate of children with medulloblastoma still remains at only 14% to 17% [Hage et al., 2002]. Consequently, new clinical approaches need to be developed in order to improve the efficiency of current treatments.

Nutraceutical approaches have recently been the focus of attempts to treat several diseases such as osteoarthritis [Moreau et al., 2003], asthma [Hosseini et al., 2001], age-related diseases [Villeponteau et al., 2000] and cancer [Park and Pezzutto, 2002]. Among these nutraceutical substances, green tea has been lauded for various beneficial health effects [Sato and Miyata, 2000]. It has also been shown that intake of green tea polyphenols inversely correlated with cardiovascular mortality, hypertension, and diabetes [Dufresne and Farnworth, 2001]. Moreover, green tea can also modulate the invasive properties of numerous tumor cells and prevent cancer [Mukhtar and Ahmad, 1999; Kuroda and Hara, 1999; Demeule et al., 2002]. Although the invasiveness of tumor cells strongly depends on their ability to migrate and to adhere to their extracellular matrix (ECM) environment, little is known about the molecular processes involved in brain cancer cell adhesion/migration or their modulation by green tea.

Cell adhesion is primarily mediated by a particular class of proteins called integrins. These are cell surface $\alpha\beta$ -heterodimeric glycoproteins of which, to date, 24

$\alpha\beta$ heterodimers formed by 8 different β and 18 α subunits are known [Brakebusch et al., 2002]. Integrins bind to various ECM proteins such as collagen, fibronectin, laminin and vitronectin, and their binding activates important intracellular signaling pathways that regulate, among other events, cell proliferation and cell migration [Juliano, 2002]. Their functions have been implicated in processes such as development, the immune response, hemostasis, and maintenance of tissue integrity. Dysregulation of integrins functions is reflected in various pathological processes by chronic inflammation, tumor invasion and metastasis [Hynes, 1992; Hemler, 1998]. Because integrins play a central role in tumor metastasis and tumor angiogenesis, they may thus represent crucial, specific targets for antitumoral treatments.

Green tea catechins, and in particular EGCG, have been demonstrated to reach the brain after oral intake [Nakagawa and Miyazawa, 1997; Suganuma et al., 1998]. One may thus assume that brain tumors such as medulloblastomas may be affected by the antitumoral activities of EGCG [Demeule et al., 2002]. In the present work, we demonstrate that EGCG can affect *in vitro* DAOY medulloblastoma cell adhesion/migration properties, and that these modulations are, in part, attributed to a differential expression of specific cell surface integrins, notably $\beta 1$ integrin. These findings suggest that EGCG could act as an efficient antitumoral agent by regulating DAOY medulloblastoma cell adhesion properties.

MATERIAL AND METHODS

Reagents: Modified Eagle's medium (MEM), TRIzol reagent, trypsin, penicillin and streptomycin were purchased from Invitrogen (Burlington, ON). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). Fibronectin was from Roche (Mississauga, ON). Type I collagen was extracted from rat tail tendon according to standard protocols [Silver and Trelstad, 1980]. Vitronectin was prepared as previously described [Yatohgo et al., 1988]. EDTA, EGC, EGCG, agarose, laminin-1, sodium dodecyl-sulfate (SDS), bovine serum albumin (BSA), and etoposide were purchased from Sigma (Oakville, ON).

Antibodies: Rat mAb 69.6.5 against α_v integrin was produced as previously described [Lehmann et al., 1994]. Mouse mAbs Gi9 (anti- α_2), C3VLA3 (anti- α_3), HP2/1 (anti- α_4), K20 (anti- β_1), SZ21 (anti- β_3) and rat mAb GoH3 (anti- α_6) were from Immunotech (Marseille, France). Mouse mAbs FB12 (anti- α_1), P1D6 (anti- α_5) and LM609 (anti- $\alpha_v\beta_3$) were from Chemicon (Temecula, CA). The mouse mAb CS β_6 (anti- β_6) was kindly provided by Dean Sheppard (University of California, San Francisco, CA). Goat anti-mouse FITC-conjugated antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Sheep anti-rat FITC-conjugated antibodies were from Sigma (St Louis, MO).

Cells and culture medium: The human DAOY medulloblastoma cell line was purchased from ATCC and was maintained in MEM containing 10% (v/v) FBS, 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were incubated at 37°C, with 95% air and 5% CO₂. Subconfluent cells were used for all experiments. All treatments were performed in serum-free MEM for 18h.

Cell adhesion assays: Adhesion assays were performed as previously described [Rigot et al., 1998]. Briefly, adhesion wells were coated with purified ECM protein solutions for 2h at 37°C, and then blocked by adding a solution of PBS/BSA 0.5%. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, added to precoated wells and allowed to adhere to the substrata for 2h at 37°C. After washing, adherent cells were stained with a solution of 0.1% crystal violet/20% (v/v) methanol and lysed with 1% SDS. Spectrophotometric absorbance was then measured at 600 nm.

Migration assays: The lower surface of Transwell filter inserts (8µm pore size; Costar, Acton, MA) was precoated with type I collagen, laminin-1, vitronectin or fibronectin, at a concentration of 10 µg/ml, for 2h at 37°C. The Transwells were then assembled in a 24-well plate (Falcon), the lower chambers were filled with 600 µl of serum-free medium, and 100 µl of DAOY medulloblastoma cells (5×10^5 cells/ml) were inoculated into the upper chamber of each transwell. The plate was then left at 37°C in 95% air and 5% CO₂ for 2h. Cells that had migrated to the lower surface of the filters were fixed, stained with 0.1% crystal violet/20% (v/v) methanol and counted. Five random microscopic fields were counted at a magnification of $\times 200$.

Flow cytometry analysis: Cell surface expression of integrin subunits on DAOY cells was measured by flow cytometry [Rigot et al., 1998]. Subconfluent cells were harvested and resuspended in MEM containing 20% FBS and 1% BSA. The single cell suspension (10^6 cells/ml) was incubated for 1 hour at 4°C in the presence of anti-integrin mAbs (10 µg/ml each). Cells were rinsed once and then incubated in the same buffer with an appropriate secondary FITC-conjugated antibody for 1h at 4°C. After washing, cells were fixed with 1% paraformaldehyde and cell-bound fluorescence was quantified using a Becton-Dickinson FACScan flow cytometer. To

standardize our experimental conditions, all samples were prepared with the same cell suspension.

Fluorimetric caspase-3 assay: Cells which had been treated with either 50 μ M etoposide, 10 or 20 μ M of EGC or EGCG were collected and washed in cold PBS. Cells were lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4°C, and the lysates were further clarified by centrifugation at $16,000 \times g$ for 20 min. Caspase-3 activity was determined by incubation with 50 μ M caspase-3 specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-AFC (Ac-DEVD-AFC) in 50 mM HEPES pH 7.4, 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio-1-propane-sulfonate], 5 mM DTT, and 1mM EDTA (assay buffer) on 96-well plates. The release of AFC ($\lambda_{\text{ex}} = 400\text{nm}$, $\lambda_{\text{em}} = 505 \text{ nm}$) was monitored for at least 20 min at 37°C on a SpectraMAX Gemini fluorescence plate reader (Molecular Devices).

Total RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was extracted from cultured DAOY medulloblastoma cells using the TRIzol Reagent. RT-PCR reactions were performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq Kit from Invitrogen (Burlington, ON). RT-PCR reactions were performed with specific oligonucleotide primers derived from human sequences [Bell et al., 2001], and PCR conditions were optimized so that the gene products were obtained during the exponential phase of the amplification. PCR products were resolved on 1% (w/v) agarose gels containing 1 μ g/ml ethidium bromide.

RESULTS

EGCG specifically decreases DAOY cell migration on collagen. We previously demonstrated the capability of EGCG to inhibit several key processes involved in the invasive properties of brain tumor-derived U-87 glioblastoma cells [Annabi et al., 2002]. These included inhibition of soluble proMMP-2 and inhibition of MT1-MMP mediated cell invasion. Consequently, we sought to learn whether EGCG could also affect the migratory potential of medulloblastoma DAOY cells, a highly invasive pediatric brain tumor-derived cell line. We first determined the ECM specificity and basal migratory potential of DAOY cells using modified Boyden chambers with porous filters coated with purified ECM proteins. We found that DAOY cells displayed a stronger migratory potential towards collagen and vitronectin than to any other ECM protein, with laminin being the matrix protein towards which DAOY migrated the least (Fig.1a). EGCG treatment of DAOY cells specifically inhibited cell migration on collagen in a dose-dependent manner, while no significant effect was observed on the other three substrates tested (Fig.1b). A dose of 10 μ M was found to inhibit approximately by half DAOY cell migration. Furthermore, epigallocatechin (EGC), a catechin that we previously reported not to affect U-87 glioma cell migration [Annabi et al., 2002] did not affect DAOY migration on any of the ECM substrates tested. We conclude that EGCG specifically inhibits DAOY cell migration on collagen and that the gallate moiety of EGCG may, in part, be a crucial structural feature involved in this process and that should be further investigated.

DAOY cell adhesion on collagen is increased by EGCG treatment. Cell migration is intimately associated with cell adhesive properties. We thus assessed the effects of EGCG and EGC on DAOY cell adhesion. As treatment with 10 μ M EGCG significantly reduced DAOY cell migration, we used this concentration of polyphenol for further experiments. We performed adhesion assays with various concentrations

of different purified ECM proteins. As shown in Figure 2, DAOY cells adhered more readily to collagen and vitronectin than to fibronectin or laminin. Half-maximal binding was achieved at 0.25 $\mu\text{g/ml}$ for collagen and 0.35 $\mu\text{g/ml}$ for vitronectin, while the corresponding values for laminin and vitronectin are 2.0 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$, respectively. EGCG treatment of DAOY increased cell adhesion and affinity to collagen, without significantly affecting cell adhesive properties on fibronectin, vitronectin or laminin (Fig.2). No effect of EGC treatment on DAOY cell adhesion was observed with collagen or the other matrices (not shown).

Green tea catechins EGCG and EGC do not trigger DAOY cell apoptosis.

When used above 10 μM , green tea polyphenols can trigger pro-apoptotic events in several cell types [Islam et al., 2000]. To address this issue, we measured the activation of caspase-3, the activity of which has emerged as a powerful marker for cells undergoing apoptosis [Abu-Qare and Abou-Dania, 2001]. As a positive control, cells were treated with etoposide, a pro-apoptotic agent and a strong inducer of caspase-3. As expected, etoposide strongly induced caspase-3 expression (6.3-fold), while neither EGCG nor EGC triggered, at the two concentrations used, any activation of caspase-3 (Fig.3). Moreover, no necrosis was observed in EGC- or EGCG-treated DAOY cells when cell viability was tested by a trypan blue exclusion assay (not shown). This lack of pro-apoptotic effects was noticed in other cell lines such as U-87 glioma [Annabi et al. 2002], endothelial cells [Lamy et al., 2002], and mesenchymal stem cells [Annabi et al., 2003], but does not preclude that viability of other cells be as sensitive to EGCG. This crucial observation further emphasizes the efficacy and immediate safe use of low green tea catechin concentrations in future clinical treatments.

$\beta 1$ integrin subunit mediates DAOY cell adhesion to collagen. The integrins are the best-characterized cell surface adhesion receptors. These transmembrane proteins play a crucial role in several steps in cell motility, including cell adhesion

and migration. Because EGCG can modulate these two cellular processes, we first characterized the integrins present at the DAOY cell surface using flow cytometry. A broad spectrum of integrin subunits was expressed at the DAOY cell surface. Fluorescence data clearly show that integrin $\alpha v\beta 3$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, αv , $\beta 1$ and $\beta 3$ subunits were expressed in DAOY cells, while levels of $\alpha 5$ and $\beta 6$ subunit expression were very low (Fig.4a). Such spectrum reflects the many cellular processes that may be tightly regulated by these integrins and which include: growth, death (apoptosis), adhesion, migration, and invasion by activating several signaling pathways. These integrins are thus attractive targets for antitumoural drug design aimed at inhibiting integrin activity, which may thus be useful in controlling the metastatic spread of cancer.

Since EGCG only affected cell migration and adhesion on collagen, we hypothesized that this catechin could modulate the expression of the specific integrin subunits responsible for cell adhesion on that particular matrix. Since various integrin subunits can interact with collagen, we tried to determine which were involved in the collagen adhesion process in DAOY. We thus performed experiments with specific integrin blocking antibodies. We incubated DAOY cells only with the antibodies directed towards the four main integrin subunits known to interact with collagen (i.e. $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ integrin) and performed adhesion assays on collagen. While functional blocking of $\alpha 1$ and $\alpha 3$ integrins did not affect DAOY cell adhesion to collagen, blocking of $\alpha 2$ subunits lead to a 40% decrease in cell adhesion to collagen (Fig.4b). Moreover, when DAOY were incubated in the presence of anti- $\beta 1$ antibody, cell adhesion to collagen was almost completely antagonized (Fig.4b). This strongly suggests that $\beta 1$ -containing integrins, including the integrin $\alpha 2\beta 1$, are the major receptors mediating DAOY cell adhesion to collagen.

EGCG up-regulates the gene and cell surface expression of the $\beta 1$ integrin subunit in DAOY cells. Flow cytometry analysis was performed in order to

investigate the effect of EGCG treatment on cell surface integrin expression. Once again, only $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ subunit expressions were investigated because these integrins are known to mediate cell adhesion on collagen in many cell types. As a negative control, we also monitored the expression of $\beta 3$ subunit, which does not interact with collagen. We found that $\alpha 1$, $\alpha 2$, and $\alpha 3$ integrin expression was not altered by treatment of DAOY cells with EGCG and that no variation in the expression of $\beta 3$ subunit was observed (Fig.5a). However, we observed a strong increase ($\approx 50\%$) in $\beta 1$ subunit expression, which was induced by EGCG, as demonstrated by the shift in fluorescence intensity. This increase in $\beta 1$ integrin at the cell surface of EGCG-treated DAOY cells was corroborated by the demonstration of an increase in $\beta 1$ integrin transcript levels using RT-PCR, while $\alpha 2$ and $\beta 3$ integrin transcripts remained unaffected (Fig.5b).

DISCUSSION

Medulloblastomas represent one of the most aggressive pediatric brain tumours. Currently, no treatment other than surgery exists to definitively prevent tumour development. The development of new therapeutic approaches, such as those aimed at targeting tumor neovascularization, is obviously needed. Among the anti-angiogenic agents developed and tested in the last few years, the anticancer properties of naturally occurring green tea polyphenols have shown promising potential to inhibit tumor growth and to target the invasive characteristics of cancer cells [Demeule et al., 2002]. Although the effects of EGCG, the most abundant catechin and also the main active component of green tea, on cell migration/adhesion have been recently investigated [Zhang et al., 2000; Suzuki and Isemura, 2000], no studies have examined the effect of EGCG on the modulation of the specific cell adhesion receptors involved. We thus tested the effect of EGCG on several motility processes of DAOY medulloblastoma cells. We demonstrate that EGCG affected both DAOY in vitro cell migration and ECM adhesion properties. Specifically, we show that DAOY cells express high levels of the $\beta 1$ integrin subunit and that attachment of DAOY cells to type-I collagen is completely abolished by function-blocking antibodies against the $\beta 1$ integrin subunit. We also demonstrate that EGCG treatment specifically upregulated both the gene and protein expression of $\beta 1$ integrin subunit in DAOY cells. Our study thus provides cellular and molecular evidence that cell surface integrins may represent a novel molecular target for green tea catechin EGCG in DAOY cells. Further in vivo experimentation will have to be required in order to demonstrate EGCG's efficacy as an anti-tumor agent in patients afflicted with pediatric brain tumors.

Since cell migration can be considered as a finely regulated equilibrium between adhesion and de-adhesion of cells [Friedl and Brocker, 2000], a differential

expression of cell adhesion receptors induced by EGCG may thus impact on DAOY cell migration. Such an effect of cell surface integrin expression on cell motility has been suggested [Palecek et al., 1997], and a strict correlation can thus be established between increased adhesion and decreased cell migration in DAOY cells upon EGCG treatment. One can thus safely postulate that the inhibition of DAOY cell migration can be explained by the upregulation of the $\beta 1$ integrin subunit, which would favor the immobilization of DAOY cells on type-I collagen.

Integrin receptors involved in collagen binding such as the $\beta 1$ integrin are known to associate with different α subunits. It is tempting to suggest that the functionality of heterodimers that could form through the association with specific α integrin subunits could also be modulated by EGCG in DAOY cells. Since only adhesion to collagen was affected by EGCG, and because $\alpha 1$, $\alpha 2$ and $\alpha 3$ integrin subunit expression was not modulated, we cannot exclude the possibility that the expression of other α integrin subunits such as $\alpha 10$ and $\alpha 11$, both reputed to interact with collagen, could be modulated by EGCG. Moreover, in addition to cell surface expression, integrin adhesive functions can be regulated by changes either in the affinity of the individual receptors for their respective ligands or in integrin avidity (i.e. integrin clustering) which may allow more efficient interaction between cells and ECM [Gonzalez-Amaro and Sanchez-Madrid, 1999]. Therefore, although EGCG treatment of DAOY cells resulted in the specific upregulation of the $\beta 1$ integrin subunit, an increase of affinity or avidity in association with other α integrin subunits cannot be excluded.

Many of the documented effects of EGCG treatments on cell migration have mainly been correlated to the inhibitory effect of EGCG on matrix metalloproteinases (MMP), a major class of proteolytic enzymes also actively implicated in ECM

remodeling and which play a crucial role in the cell migration process [Dell'Aica et al., 2002; Annabi et al., 2002; Islam et al., 2002]. Thus, the inhibition of DAOY cell migration that we report in the present study may not be solely attributed to an upregulation of collagen adhesion receptors. MMPs participate in the invasion process and, consequently, are highly involved in cell migration [Visse and Nagase, 2003]. Among them, the MMP-2 and MMP-9 collagenases have been shown to display a strong pro-migratory activity [Hornebeck et al., 2002]. We have already demonstrated that EGCG inhibited proMMP-2 secretion in U-87 glioblastoma cells [Annabi et al., 2002]. Interestingly, we also observed that proMMP-2 secretion was partially inhibited by EGCG treatment in medulloblastoma-derived DAOY cells (unpublished data). We thus can not exclude a potential role of EGCG-mediated inhibition of MMP-2 secretion in the modulation of DAOY cells motility processes.

Although adhesion is the primary function of integrins, they are also important signaling molecules. Integrins regulate intracellular signaling pathways controlling cell migration and invasion, cytoskeletal organization, force generation and survival [Giancotti and Ruoslahti, 1999; Parise et al., 2000]. Whether EGCG interferes with any of the intracellular integrin-mediated signaling in DAOY cells is currently under investigation and still remains to be established. It is worth noting that recent evidence suggests that the PI3K/Akt pathway, a crucial integrin-mediated signaling pathway involved in apoptosis, is affected by EGCG [Waltner-Law et al., 2002]. This pathway particularly involves the functions of an integrin-linked kinase (ILK), which promotes cell proliferation through the activation of PKB/Akt anti-apoptotic signaling pathway [Cruet-Hennequart et al., 2003; Troussard et al. 2003]. Since integrins have no intrinsic enzymatic activity, it will be crucial to assess whether other integrin-associated proteins, such as the src-family kinases (c-src, fyn, yes), FAK, PKA and Pyk-2 [Miranti and Brugge, 2002], may also be targeted by EGCG. Ultimately, EGCG may also have a dual role in targeting both medulloblastoma-derived cancer and endothelial cells. Interestingly, $\beta 1$ integrins, which are not unique

to angiogenic endothelial cells, may also support most of the adhesion, migration, and signalling events essential for angiogenesis [Luscinskas and Lawler, 1994]. Current studies in our laboratory are underway to explore the above transduction pathways that may be modulated by EGCG. Moreover, special focus will be directed towards the identification of the $\beta 1$ integrin(s) mediating endothelial/cancer cell adhesion, migration, and signalling events required for angiogenesis and metastasis.

Suppressing tumor growth by antiangiogenesis and vascular targeting strategies has led to the recent development of a large number of natural and synthetic antiangiogenic compounds [Ranieri and Gasparini, 2001]. These include inhibitors of VEGF, VEGF-R2, MMP and integrin antagonists. Experimental evidence suggests that antiangiogenic treatments may be more effective in suppressing tumor growth when used in combination with conventional chemotherapy, immunotherapy, or radiotherapy [Koukourakis, 2001]. Interestingly, recent evidence from our laboratory has demonstrated the pleiotropic effects of green tea catechin EGCG on MMP-mediated events in glioblastoma cells [Annabi et al., 2002], and on suppressing tubulogenesis and VEGF-R2 receptor mediated effects in endothelial cells [Lamy et al., 2002]. Moreover, we have shown that treatment of human umbilical vein endothelial cells (HUVEC) with doses of EGCG equivalent to those found in human plasma after oral administration (2-5 μ M), combined with low-doses of ionizing radiation, affected the cell adhesive properties of HUVEC and suppressed tubulogenesis [Annabi et al., unpublished data]. In light of the data obtained in the present study, it is thus tempting to suggest that low-dose metronomic administration of naturally occurring green tea polyphenol may provide, in combination with radiotherapeutic modalities, a new perspective in treating pediatric patients with medulloblastoma.

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FIGURE LEGENDS

Figure 2.1: Green tea catechin EGCG specifically inhibits DAOY cell migration on collagen. (A) Haptotaxis assays were performed in a transwell chamber, the lower surface of which was coated with either type I collagen, fibronectin, vitronectin or laminin-1 at 10 μ g/ml. (B) DAOY cells were treated (or not) with increasing concentrations of EGCG (●) or EGC (o) for 18h. Single cell suspensions (5×10^4) were seeded onto the upper surface of transwells and allowed to migrate for 2 h. Cells that migrated to the bottom surface were stained as described in the Methods section. Data are presented as the mean number ($\pm$ SEM) of cells migrated per field of microscope (magnification $\times 200$) from at least three independent experiments. ★ indicates statistically significant differences ($P < 0.05$) compared with the EGC treatment of DAOY using Student's t-test.

Figure 2.2: Green tea catechin EGCG specifically increases DAOY cell adhesion to collagen. DAOY cells were treated as described in the legend to Fig.1 for 18h with either carrier (o) or 10 μ M EGCG (●). The cells were harvested with a 0.53 mM EDTA/PBS solution and washed twice with adhesion buffer. Cells (5×10^4) were seeded on a 96-well plate, previously coated with various purified ECM proteins. Cells were allowed to adhere for 2h at 37°C. Data shown are mean $\pm$ SEM of at least three independent experiments performed in triplicate.

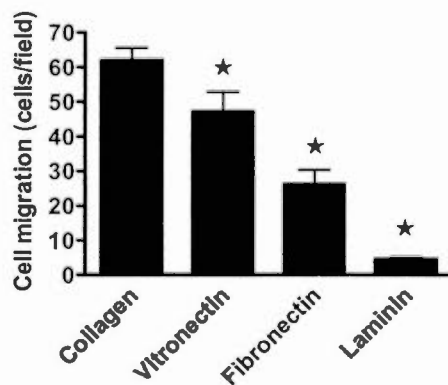
Figure 2.3: Green tea catechins EGCG and EGC do not induce pro-apoptotic activity in DAOY cells. Fluorimetric caspase-3 activation assays have been performed using cells which were treated for 18h with either 50 μ M etoposide, 10 and 20 μ M EGCG or EGC. ★ indicates statistically significant differences ($P < 0.05$) compared with control (untreated DAOY) cells using Student's t-test.

Figure 2.4: Expression of integrin subunits at the DAOY cell surface. (A) Flow cytometric analysis was performed using antibodies directed towards specific integrin subunits as described in the Methods section. (B) Adhesion assays have been performed with DAOY cells preincubated with blocking antibodies directed against various integrin subunits. Cells were allowed to adhere on 10 $\mu\text{g/ml}$ collagen for 2h at 37°C. All assays were performed in triplicate and the results ($\pm$ SEM) of three independent experiments are shown.

Figure 2.5: Green tea catechin upregulates $\beta 1$ integrin subunit expression at the DAOY cell surface. DAOY cells were treated (or not) with 10 μM EGCG for 18h. (A) Expression of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$ and $\beta 3$ at the cell surface was monitored by flow cytometry as described in the Methods section (shaded area: control; lined curve: EGCG treatment). (B) Total RNA was isolated from untreated or EGCG-treated DAOY cells and $\beta 1$, $\alpha 2$ and $\alpha 3$ integrin subunits gene products were amplified by RT-PCR.

Figure 1: Green tea catechin EGCG specifically inhibits DAOY cell migration on collagen.

A.



B.

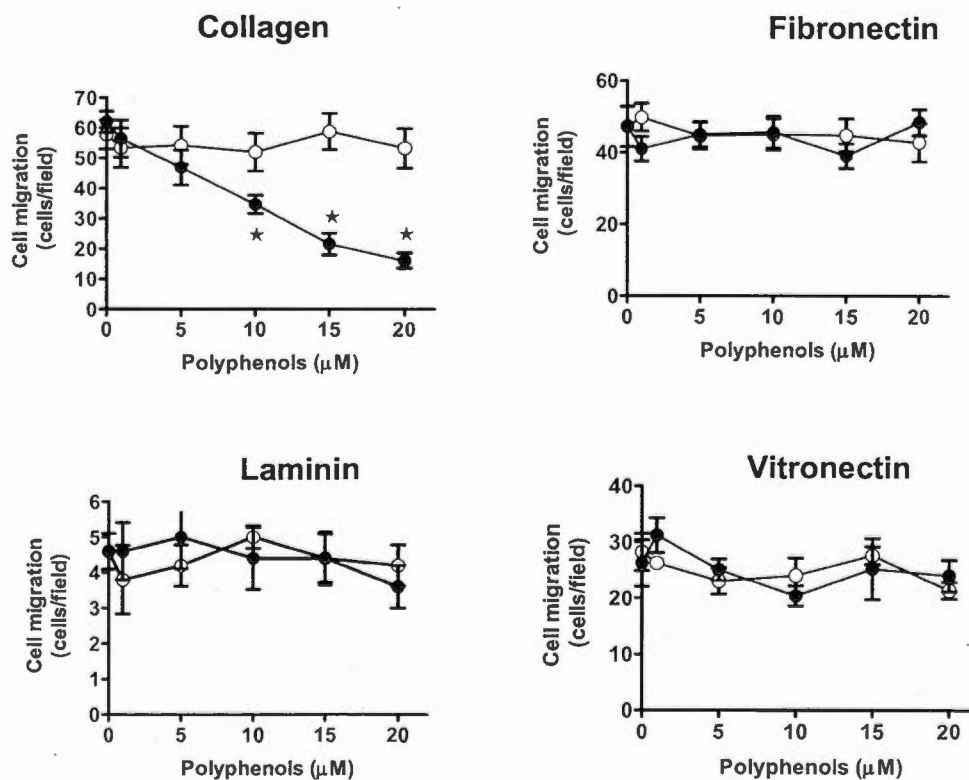


Figure 2: Green tea catechin EGCG specifically increases DAOY cell adhesion to collagen

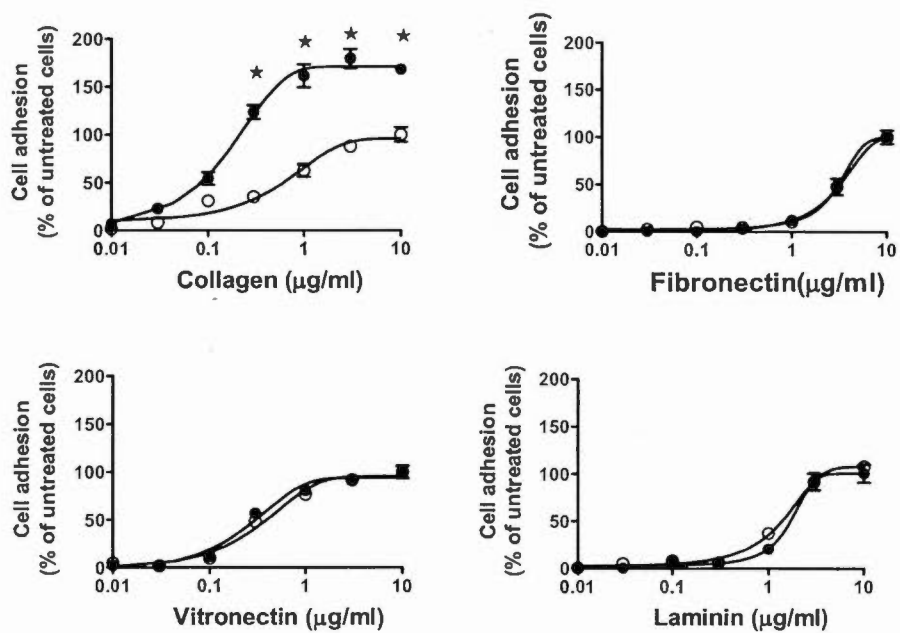


Figure 3: Green tea catechins EGCG and EGC do not induce pro-apoptotic activity in DAOY cells

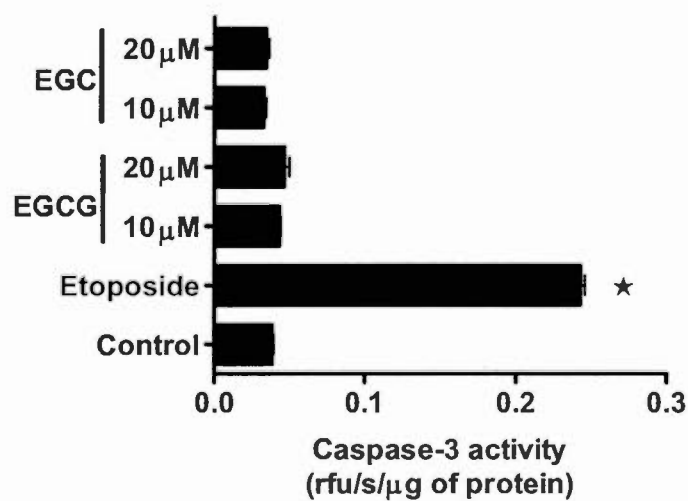
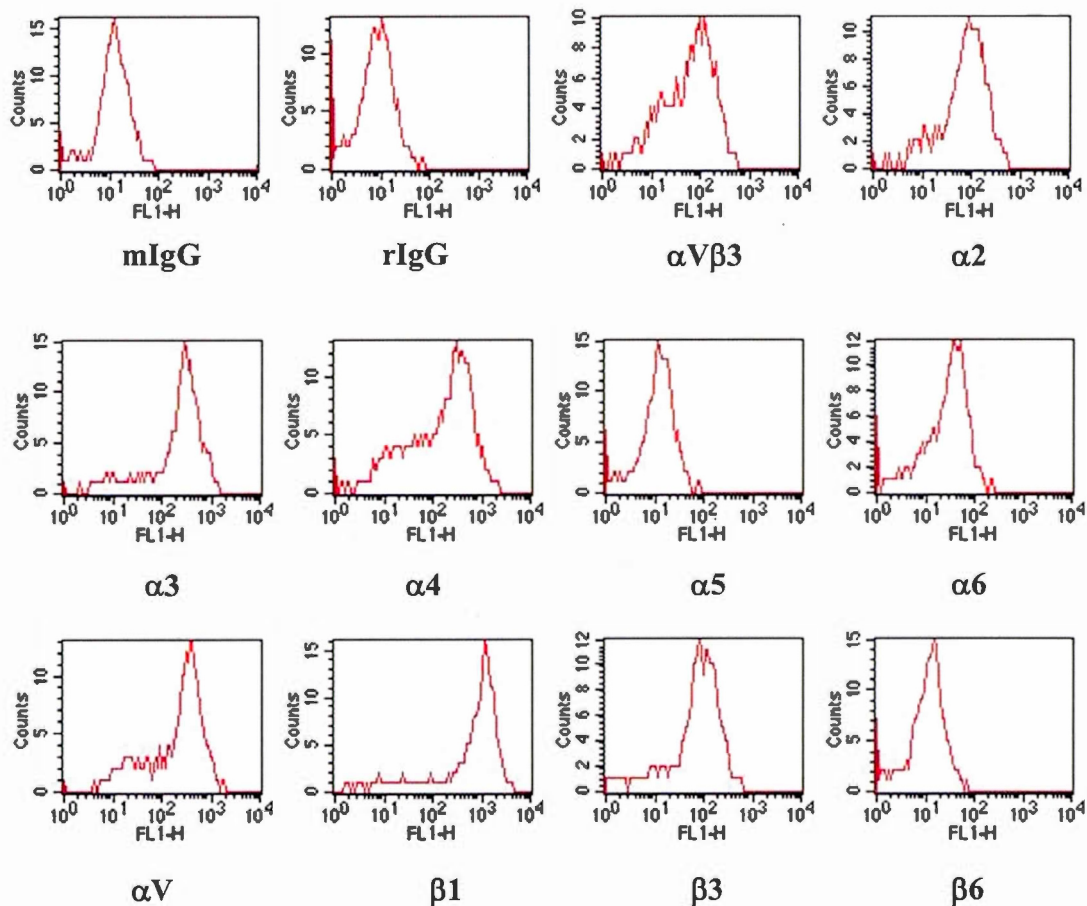


Figure 4: Expression of integrin subunits at the DAOY cell surface

A.



B.

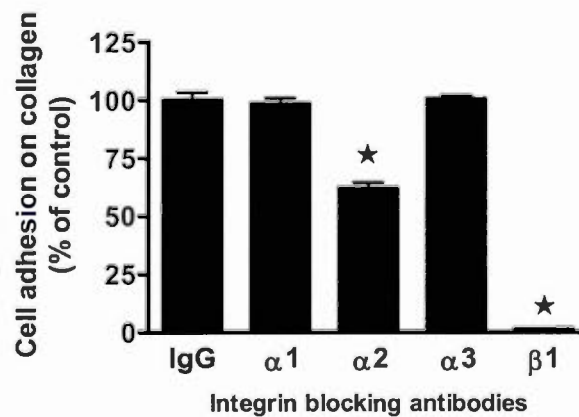
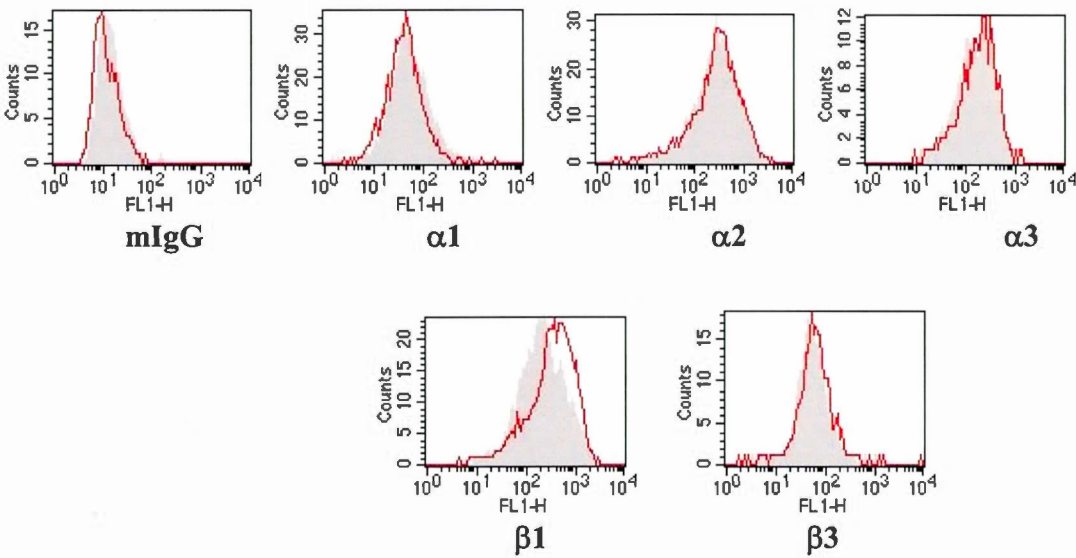
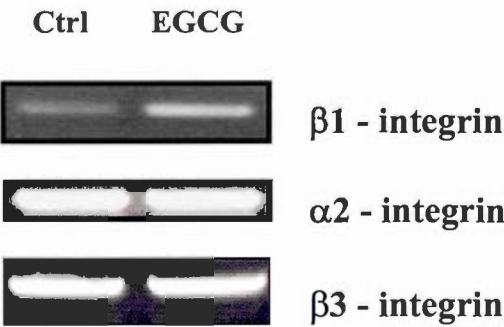


Figure 5: Green tea catechin upregulates $\beta 1$ integrin subunit expression at the DAOY cell surface

A.



B.



Article 2 : Lebectin, a *Macrovipera lebetina* venom-derived C-type lectin, inhibits angiogenesis both *in vitro* and *in vivo*

Running title: Lebectin displays antiangiogenic activity.

Anthony Pilorget^{1,2}, Magali Conesa¹, Sameh Sarray³, Jonathan Michaud-Levesque², Salma Daoud³, Kwang Sik Kim⁵, Michel Demeule², Jacques Marvaldi¹, Mohamed El Ayeb³, Naziha Marrakchi^{3,4}, Richard Béliveau² and José Luis^{1*}

¹CNRS FRE 2737, Faculté de Pharmacie, 27, bd Jean Moulin, 13385 Marseille, Cedex 5, France, ²Laboratoire de Médecine Moléculaire, Centre de Cancérologie Charles-Bruneau, Hôpital Sainte-Justine-UQAM, Montreal, Quebec, Canada, ³Laboratoire des Venins et Toxines, Institut Pasteur de Tunis, 13, place Pasteur, BP 74, 1002 Belvédère, Tunis, Tunisia, ⁴Faculté de Médecine de Tunis, 9 Rue Professeur Zouheir Essafi, Tunisia, and ⁵Division of Pediatric Infectious Diseases, Johns Hopkins University, School of Medicine, Baltimore, Maryland, USA.

* To whom correspondence and reprint requests should be directed:

CNRS FRE 2737, Faculté de Pharmacie

27, bd Jean Moulin, 13385 Marseille, Cedex 5, France

Tél : (+33) 04 91 83 56 58

Fax : (+33) 04 91 83 56 53

E-mail : jose.luis@pharmacie.univ-mrs.fr

The abbreviations used are: CLP, C-type lectin protein; HBMEC, human brain microvascular endothelial cell; ECM, extracellular matrix; CAM, chorioallantoic membrane.

Keywords: Angiogenesis, C-type lectin, integrin, snake venom, *Macrovipera lebetina*

CONTRIBUTION

Ce deuxième article a permis la caractérisation des propriétés antiangiogéniques de la lébectine, une lectine de type C isolée du venin de la vipère *Macrovipera lebetina*. En utilisant les cellules endothéliales cérébrales humaines (HBMEC) comme modèle *in vitro*, nous avons démontré que cette lectine présente des effets antiangiogéniques puissants. Nous avons aussi caractérisé les effets antiangiogéniques de la lébectine *in vivo*. Du point de vue moléculaire, nous avons démontré que la lébectine inhibe les fonctions d'adhérence des intégrines $\alpha 5\beta 1$ et αV . Cet article est le second à décrire le potentiel antiangiogénique d'une lectine de type C isolée d'un venin de serpent.

J'ai réalisé les expériences, en collaboration avec le Dr Magali Conesa de l'unité CNRS FRE 2737 de Marseille, le Dr Sameh Saray de l'Institut Pasteur de Tunis et Jonathan Michaud Levesque, étudiant au doctorat dans le laboratoire du Dr Béliveau à Montréal. Ces travaux ont été réalisés sous la supervision directe de mes deux co-directeurs, le Dr Richard Béliveau et le Pr José Luis. J'ai rédigé la totalité de ce manuscrit. La relecture de cet article a été faite par le Pr José Luis à Marseille, le Dr Richard Béliveau et le Dr Michel Demeule à Montréal et le Dr Naziha Marrackchi à Tunis. Cet article a été accepté pour publication dans la revue scientifique *Journal of Cellular Physiology* en octobre 2006.

RÉSUMÉ EN FRANÇAIS

Les intégrines jouent un rôle majeur dans les processus de mobilité cellulaire des cellules endothéliales au cours de l'angiogenèse. Elles représentent donc des cibles potentielles pour le développement de nouveaux agents antiangiogéniques. Les venins de serpents contiennent naturellement une grande variété de protéines qui peuvent affecter les interactions entre les intégrines et leurs ligands. Récemment, il a été démontré que les lectines de type C sont des modulateurs importants de la fonction des intégrines. Dans cette étude, nous avons étudié l'activité antiangiogénique de la lébectine, une lectine de type C isolée du venin de la vipère *Macrovipera lebetina*. Les cellules endothéliales cérébrales humaines HBMEC expriment les intégrines vasculaires $\alpha v\beta 3$, $\alpha v\beta 5$ et $\alpha 5\beta 1$, de même que les intégrines $\alpha 2$, $\alpha 3$, $\alpha 6$ et $\beta 4$. Nos résultats montrent que la lébectine est un puissant inhibiteur ($IC_{50} \approx 0.5$ nM) de l'adhérence et de la migration des cellules HBMEC sur fibronectine, par le blocage de la fonction d'adhérence des intégrines $\alpha 5\beta 1$ et αV . De plus, la lébectine inhibe fortement la tubulogenèse sur Matrigel™ des cellules HBMEC, de même que leur prolifération. Finalement, en utilisant des tests *in vivo* de mesure de l'angiogenèse (membrane chorioallantoïdienne de poulet et implantation sous-cutanée de Matrigel™), nous démontrons que la lébectine bloque fortement l'angiogenèse *in vivo*. La lébectine représente donc une nouvelle lectine de type C aux propriétés antiangiogéniques importantes et un nouvel espoir thérapeutique pour le traitement des maladies à composante angiogénique.

ABSTRACT

Integrins play an essential role in endothelial cell motility processes during angiogenesis and thus present interesting targets for the development of new anti-angiogenic agents. Snake venoms naturally contain a variety of proteins that can affect integrin-ligand interactions. Recently, the C-type lectin proteins (CLPs) have been characterized as efficient modulators of integrin functions. In this study, we investigated the anti-angiogenic activity of lelectin, a newly discovered CLP from *Macrovipera lebetina* venom. Human brain microvascular endothelial cells (HBMEC), used as an *in vitro* model, express $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins, as well as the α_2 , α_3 , α_6 and β_4 subunits. Our data show that lelectin acts as a very potent inhibitor ($IC_{50}\approx 0.5$ nM) of HBMEC adhesion and migration on fibronectin by blocking the adhesive functions of both the $\alpha_5\beta_1$ and α_v integrins. In addition, lelectin strongly inhibits both HBMEC *in vitro* tubulogenesis on Matrigel™ ($IC_{50}=0.4$ nM) and proliferation. Finally, using both a chicken CAM assay and a Matrigel™ Plug assay in nude mice, our results show that lelectin displays potent anti-angiogenic activity *in vivo*. Lelectin thus represents a new C-type lectin with anti-angiogenic properties with great potential for the treatment of angiogenesis-related diseases.

INTRODUCTION

Angiogenesis is the formation of new capillaries from preexisting blood vessels (Folkman 2004). The angiogenic process is fundamental to normal healing, reproduction and embryonic development (Zadeh and Guha 2003). It has also been implicated in the pathogenesis of a wide variety of disorders, including primary and metastatic tumors, aneurysms, arteriovenous malformations and cavernous malformations (Harrigan 2003). The process of angiogenesis is complex, typically consisting of enzymatic degradation of the basement membrane, vascular endothelial cell migration into perivascular space, proliferation and alignment to form capillary-like structures, and new vessel formation (Folkman 2001). Although growth factors stimulate new blood vessel growth and survival (Folkman 1995b, 2004), adhesion to the extracellular matrix (ECM) also regulates endothelial cell survival, proliferation and motility during new blood vessel formation (Li et al. 2003). At the molecular level, interactions between vascular cells and ECM are mainly mediated by integrins, a family of transmembrane proteins that link the ECM to the actin cytoskeleton within the cell (Hwang and Varner 2004).

Integrins are heterodimeric glycoproteins consisting of non-covalently associating α and β subunits. To date, 24 $\alpha\beta$ heterodimers, formed by combinations of 8 β and 18 α subunits, are known (Brakebusch et al. 2002). They are involved in various processes such as development, immune response, hemostasis and maintenance of tissue integrity. Integrins also participate in various pathological processes such as chronic inflammation, tumor invasion and metastasis (Hynes 1992; Hemler 1998). In addition to their role in cell adhesion, integrins relay molecular signals from the cellular environment which influence cell shape, survival, proliferation and gene transcription (Guo and Giancotti 2004). More recently, integrins have also been shown to be pivotal molecules in the angiogenic process

(Hodivala-Dilke et al. 2003; Hwang and Varner 2004; Stupack and Cheresch 2004). Consequently, they represent a target of choice for antiangiogenic therapy.

Snake venoms have been shown to possess unique components able to affect cell-ECM interactions. In particular, some have been demonstrated to alter integrin adhesive properties (Chung et al. 2003; Yang et al. 2005b). Among these molecules, snake venom disintegrins have been extensively studied (McLane et al. 2004). Another class of peptides, identified as C-type lectin proteins (CLPs), has also been reported to block cell-ECM interactions (Sarray et al. 2001; Sarray et al. 2004). CLPs represent a large family of Ca^{++} -dependent lectins that share primary structural homology in their carbohydrate-recognition domains (Morita 2004). Snake venom CLPs are proteins of about 30 kDa, comprised of two associated subunits, which display varied, distinct biological activities (Goldstein and Hayes 1978; Ogawa et al. 2005). CLPs were first characterized for their role in affecting immune system functions, such as inflammation and immunity against tumors and virally-infected cells. Several CLPs have also been shown to strongly modulate the platelet aggregation process via the interactions between von Willebrand factor and platelet GPIb (Peng et al. 1993; Yoshida et al. 1993; Yeh et al. 2000; Tai et al. 2004). However, their role in angiogenesis, through their anti-integrin properties, remains to be elucidated.

The ability of some *Macrovipera lebetina* venom-derived CLPs to inhibit tumor cell migration through their anti-integrin properties has already been demonstrated (Sarray et al. 2001; Sarray et al. 2004). Consequently, we investigated how lebelectin, a previously characterized CLP from *Macrovipera lebetina* venom (Sarray et al. 2004), can modulate the angiogenic properties of endothelial cells *in vitro* and *in vivo*. As an *in vitro* model, we used human brain endothelial cells (HBMEC) to represent this important therapeutic target for the specific treatment of brain tumors which do not respond to conventional treatments, such as chemotherapy

and radiotherapy. We compared the antiangiogenic effects of lebectin with those of lebecetin, another *Macrovipera lebetina* venom-derived CLP (Sarray et al. 2001; Sarray et al. 2003). Our results strongly suggest that lebectin is a potent inhibitor of both *in vitro* and *in vivo* angiogenesis. Lebectin could thus be used as an antiangiogenic agent in the treatment of angiogenesis-related diseases.

MATERIAL AND METHODS

Chemicals: Venom was collected from *M. lebetina* snakes in the serpentarium of the Institut Pasteur, Tunis. Lebecetin and lebecetin were purified as previously described (Sarray et al. 2001; Sarray et al. 2003; Sarray et al. 2004). Trypsin, penicillin and streptomycin were from Invitrogen (Burlington, ON). Fibronectin was from Roche (Mississauga, ON). Type-1 collagen was extracted from rat tail tendon by a standard method (Silver and Trelstad 1980). Vitronectin was prepared as previously described (Yatohgo et al. 1988). EDTA, laminin-1, sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Matrigel™ was from BD Biosciences Labware (Bedford, MA).

Cells and culture media: RPMI 1640 medium was from Gibco (Burlington, ON) and heat-inactivated fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT). Immortalized human brain microvascular endothelial cells (HBMEC) were maintained in RPMI 1640, 10 % heat-inactivated FBS, 10 % NuSerum (BD Biosciences Labware, Bedford, MA), ECGS (endothelial cell growth supplement) (30 µg/ml), heparin (5 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids, vitamins, penicillin and streptomycin (100 U/ml). Cells were incubated at 37°C, air/CO₂ (95 %/5 %).

Flow cytometry analysis: Cell surface expression of integrin subunits on HBMEC was determined by flow cytometry as described (Rigot et al. 1998). As a control, non-specific immunoglobulin G (IgG) was used for each experiment. To standardize our experimental conditions, all samples were prepared using the same cell suspension. Rat mAb 69.6.5, against α_v integrin, was produced as previously described (Lehmann et al. 1994). Mouse mAbs Gi9 (anti- α_2), C3VLA3 (anti- α_3), HP2/1 (anti- α_4), Lia1/2 (anti- β_1), SZ22 (anti- α_{IIb}) and rat mAb GoH3 (anti- α_6)

were from Immunotech (Marseille, France). Mouse mAbs FB12 (anti- $\alpha 1$), P1D6 (anti- $\alpha 5$), JBS5 (anti- $\alpha 5\beta 1$), LM609 (anti- $\alpha v\beta 3$), P1F6 (anti- $\alpha V\beta 5$), 10D5 (anti- $\alpha V\beta 6$) and 3E1 (anti- $\beta 4$) were from Chemicon (Temecula, CA). Goat anti-mouse FITC-conjugated antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Sheep anti-rat FITC-conjugated antibodies were from Sigma (St Louis, MO).

Covalent crosslinking and co-immunoprecipitation. HBMEC cell monolayers were incubated with 50 $\mu\text{g/ml}$ of lebeectin or lebecetin in RPMI containing 15 mM Hepes, pH 7.3 and 0.1% BSA. After 5 h at 4°C, cells were washed twice with ice-cold PBS. 2 ml PBS were added to cells and the reaction was initiated by adding 40 μl 100 mM DSP, dissolved in dimethylsulfoxide just before use. The reaction was performed at room temperature and was stopped 20 min later by the addition of 1 ml PBS containing 60 mM ammonium acetate. Cells were rinsed twice with PBS and then lysed in 1 ml 50 mM Tris-HCl pH 8, 200 mM NaCl and 1 % Triton X100 (RIPA buffer) containing 0.5 % BSA and a mixture of proteinase inhibitors (1 mM PMSF, 500 U/ml aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 μM pepstatin, 1 mM iodoacetamide and 1 mM ortho-phenantrolin). Clarified cell lysates were incubated with 5 μl of anti-lebecetin antiserum (which recognizes both lebecetin and lebeectin) overnight at 4°C. After addition of protein G-agarose beads and incubation for 1 hour, the suspension was centrifuged and pellets were washed 3 times with RIPA buffer, 3 times with RIPA buffer/500 mM NaCl and once with PBS. Immunoprecipitated proteins were solubilized in Laemmli sample buffer, heated at 100°C for 5 min. and submitted to SDS-PAGE under reducing conditions. The gel was then blotted onto a nitrocellulose sheet and probed overnight at 4°C with anti- αv or anti- $\alpha 5$ antibodies (1:1000). Blots were then revealed by a light-based ECL system using HRP-conjugated secondary antibodies.

Cell adhesion assays: Adhesion assays were performed as previously described (Rigot et al. 1998). Briefly, 96-well plates were coated with purified ECM protein solutions for 2 hours at 37°C and blocked with a solution of PBS/0.5 % BSA. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, added to precoated wells, and allowed to adhere to the substrata for 2 hours at 37°C in serum-free medium. After washing, adherent cells were fixed with 3.7 % formaldehyde, stained with a solution of 0.1 % crystal violet/MetOH 20% and lysed with 1% SDS. Absorbance was then measured at 600 nm. For adhesion assays on antibodies, 96-well plates were coated with 50 µl of rabbit anti-rat IgG (50µg/ml) overnight at 4°C. Wells were washed once with PBS and 50 µl of anti-integrin blocking antibodies were added for 5 h at 37°C. Antibodies were used as follow: anti- $\alpha 3$ (2.5 µg/ml), anti- $\alpha 5$ (1/200), anti- $\alpha 6$ (10 µg/ml), anti- αv (10 µg/ml), anti- $\alpha v\beta 3$ (10 µg/ml) and anti- $\beta 1$ (10 µg/ml). Wells were then blocked with a solution of PBS/0.5% BSA. Adhesion assays were performed as above, except that HBMEC cells were pretreated with lelectin (10 µg/ml) for 30 min at room temperature and allowed to adhere for 45 min at 37°C.

Haptotaxis migration assays: The lower face of Transwells (8 µm pore size; Costar, Acton, MA) was precoated with type I collagen, laminin-1, vitronectin or fibronectin, at a concentration of 10 µg/ml, for 2 h at 37°C. The Transwells were then assembled in a 24-well plate (Falcon), the lower chambers were filled with 600 µl of serum-free medium, and 100 µl of HBMEC (5×10^5 cells/ml) were inoculated into the upper chamber of each well. The plate was then placed at 37°C in 95% air and 5% CO₂ for 2 h. Cells which migrated to the lower surface of the filters were fixed and stained with 0.1 % crystal violet/20 % methanol (v/v). Five random microscopic fields were photographed at a magnification of $\times 200$ and counted using the Northern Eclipse analysis software (Empix Imaging inc., Mississauga, ON).

Morphogenic differentiation assay: Matrigel™ was plated in 96-well plates and allowed to gel for 2 hours at 37°C prior to cell seeding. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, at a final concentration of 10^6 cells/ml. HBMEC cells (2×10^4) were added atop the Matrigel™ in serum-free media after a 30 min pretreatment with either lebeectin or lebecetin and incubated at 37°C for 18 hours. Capillary-like structures were analyzed microscopically and photographs (50X) taken with a Retiga 1300 camera connected to a Nikon Eclipse TE2000-U microscope. The capillary-like structures formed in the gel were quantified by analysis of digitized images to determine the segment length of the capillary-like network, using Northern Eclipse analysis software (Empix Imaging Inc., Mississauga, ON). Results are expressed as a percentage of control cells.

Cell proliferation assay: HBMEC cells were seeded at 1.5×10^4 cells/cm² in the presence or absence of 5 µg/ml lebeectin or lebecetin. At daily intervals, some HBMEC cells were fixed with 3.7% formaldehyde, stained with a solution of 0.1% crystal violet/MetOH 20% and lysed with 1% SDS. Absorbance was then measured at 600 nm. All assays were performed in triplicate and data are the means of at least three independent experiments.

Chicken CAM Assay: The chicken CAMs were prepared using 8 day-old chick embryos. Filter disks were soaked in 0.9 % NaCl alone (control) or containing 0.2 µg of lebeectin or 10 µg of lebecetin. After incubation for 72 hours, CAMs were photographed with a digital camera. To quantify CAM angiogenesis, we measured the total length of vessels per photographic field. All experiments used five CAM and data are the means of three independent experiments.

In vivo Matrigel™ plug assay: All animal experiments were evaluated and approved by the Institutional Committee for Good Animal Practices (UQAM, Montréal, QC, Canada). The Matrigel™ implantation assay was based on the method of Passaniti with some modifications (Passaniti et al. 1992). Prior to injection, heparin was incubated with or without fibroblast growth factor-2 (FGF-2) for 5 min then diluted in phenol red-free Matrigel™ on ice at a final concentration of 0.0025 units/ml heparin and 250 ng/ml FGF-2. Lebectin, lebecetin or water control alone were then added and the samples were kept on ice until injection. Crl:CD-1®-nuBR nude mice (Charles River Laboratories, Lassalle, QC, Canada), between 5 and 10 weeks old, were subcutaneously injected under anesthesia in the ventral midline region of the right flank with 0.5mL of Matrigel™ alone or Matrigel™ containing FGF-2 with or without lebectin or lebecetin (5µg). After 7 days, the mice were euthanized and the Matrigel™ implant harvested, washed with PBS, and examined macroscopically for signs of hematoma. Any implant with visible signs of hematoma was excluded from analysis. The remaining implants were immediately frozen and lyophilized overnight. The weight of the dry Matrigel™ implant was determined and the implants were resuspended in 0.4 ml of 0.1% Triton X-100 for 1 h, disrupted by vigorous pipetting and centrifuged at 14000g for 15 min to remove particulates. The concentration of hemoglobin in the supernatant was then directly determined by absorbance at 405 nm and compared with a standard curve of purified hemoglobin (Sigma-Aldrich, Oakville, ON) as previously described (McMahon et al. 2001).

Statistical analysis: Results are expressed as means $\pm$ SEM and analyzed with Student's *t*-test. Only significant differences ($p < 0.05$) are indicated in the figures.

RESULTS

Basal motility properties of HBMEC

HBMEC have been previously used to study the *Escherichia coli* invasion of brain microvascular endothelial cells or as an *in vitro* model for lymphocyte transendothelial migration (Prasadarao et al. 1999; Reddy et al. 2000). However, the intrinsic motility properties of HBMEC have not been characterized to date. Consequently, we first determined the basal adhesive properties of HBMEC on various purified ECM proteins. As shown in Figure 1A, HBMEC firmly adhere to fibronectin and vitronectin but do not adhere to type 1 collagen. Moreover, these cells display a strong adhesion to laminin-1, but only at high matrix concentrations (over 5 $\mu\text{g/ml}$). We considered fibronectin to be the optimal choice of adhesion substrate for HBMEC because, at a low fibronectin concentration (1 $\mu\text{g/ml}$), the cells already display full adhesion to this ECM protein. In parallel, we used modified Boyden chambers to characterize the migratory properties of HBMEC towards various ECM purified proteins, including type I collagen, fibronectin, vitronectin and laminin-1. In agreement with the adhesion results, HBMEC displayed a high rate of migration towards fibronectin, vitronectin and laminin-1 at the concentration used (10 $\mu\text{g/ml}$). However, HBMEC did not migrate towards type 1 collagen (Fig. 1B).

Integrin expression in HBMEC

Amongst the better characterized groups of cell surface adhesion receptors are integrins. These transmembrane proteins play a crucial role during cell motility processes such as cell adhesion and migration. By flow cytometric analysis, we identified the main integrins expressed at the HBMEC cell surface. As shown in Figure 2, HBMEC express high levels of the classical endothelial integrins ($\alpha\text{V}\beta 3$, $\alpha\text{V}\beta 5$ and $\alpha 5\beta 1$), which are the major receptors for fibronectin and vitronectin in endothelial cells. They also express a large set of other integrin subunits such as $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$ and $\beta 4$. However, $\alpha 1$, $\alpha 4$, αIIb and $\alpha\text{V}\beta 6$ integrins were not detected by

FACS analysis, suggesting that their expression levels in HBMEC are low or nonexistent.

Lebectin inhibits HBMEC adhesion by blocking the adhesive functions of both $\alpha 5\beta 1$ and $\alpha V\beta 3$ integrins.

We previously reported that lebectin can inhibit tumor cell adhesion on various substrates by impairing cell/ECM interactions through an integrin-dependent mechanism (Sarray et al. 2004). Consequently, we tested the effects of various concentrations of lebectin against HBMEC cell adhesion to fibronectin and vitronectin. Low concentrations of lebectin strongly inhibited cell adhesion on fibronectin but did not affect the adhesive properties of HBMEC cells to vitronectin (Fig.3A). However, at a high concentration of lebectin (50 $\mu\text{g/ml}$), HBMEC cell adhesion to vitronectin was also impaired (Fig.3A). In parallel, we investigated the effect of lebecetin, another related *M.lebetina* venom-derived CLP (Sarray et al. 2003), under the same conditions. We observed that the inhibitory effect of lebecetin on HBMEC adhesion to fibronectin is less than that of lebectin (Fig. 3A). The IC_{50} of lebecetin-mediated inhibition of HBMEC adhesion to fibronectin is ≈ 600 -fold higher than the IC_{50} of lebectin. More precisely, the IC_{50} values for inhibition of HBMEC adhesion on fibronectin for lebectin and lebecetin are, respectively, 0.016 $\mu\text{g/ml}$ (0.52 nM) and 10.5 $\mu\text{g/ml}$ (350 nM). In contrast, HBMEC adhesion on vitronectin is unaffected by lebecetin, even at high concentrations (Fig. 3A).

HBMEC adhere on fibronectin through $\alpha 5\beta 1$ integrin and on vitronectin mainly through $\alpha V\beta 3$ integrin (Isogai et al. 2001). Because lebectin inhibits HBMEC adhesion on both fibronectin and vitronectin, we hypothesized that this C-type lectin could interact with $\alpha 5\beta 1$ and $\alpha V\beta 3$. To address this issue, HBMEC were treated with lebectin for 30 minutes and allowed to adhere on various anti-integrin antibodies. At 10 $\mu\text{g/ml}$, lebectin does not modify HBMEC adhesion on anti- $\alpha 3$, anti- $\alpha 6$ or anti- $\beta 1$ integrin antibodies (Fig.3B). However, HBMEC adhesion on an anti- $\alpha 5$ integrin antibody was almost completely inhibited (90%), which demonstrates that lebectin

can efficiently block the adhesive functions of the $\alpha 5 \beta 1$ integrin (Fig.3B). We also tested the effect of lebeectin on the HBMEC adhesion on an anti- $\alpha V \beta 3$ antibody. Our results show that lebeectin decreases HBMEC adhesion by 33% on this specific antibody (Fig.3B). These results demonstrate that lebeectin can inhibit the adhesive properties of both $\alpha 5 \beta 1$ and $\alpha V \beta 3$ integrins in HBMEC but displays a stronger effect against $\alpha 5 \beta 1$ integrin.

In order to ascertain whether the venom peptides block HBMEC adhesion through a specific interaction with integrins, HBMEC were treated with either lebeectin or lebecetin for 5 hours. Venom peptides were co-immunoprecipitated and separated by SDS-PAGE. $\alpha 5$ and αV integrins were then detected by Western blot. As shown in Figure 3C, both peptides interacted with $\alpha 5$ and αV integrins since both are co-immunoprecipitated. It should be noted that the interaction of lebecetin with αV integrin was greater than for lebeectin, despite the stronger effect of lebeectin on αV -mediated HBMEC adhesion on vitronectin. In contrast, lebeectin interacted much strongly with $\alpha 5$ than did lebecetin. This interaction pathway may explain the stronger effect of lebeectin on $\alpha 5$ -mediated HBMEC adhesion on fibronectin, compared to lebecetin.

Lebeectin inhibits HBMEC migration on fibronectin.

Cell migration can be considered as a finely regulated process including successive steps of cell adhesion and deadhesion. Because lebeectin potently blocks HBMEC cell adhesion on fibronectin, we also examined its effects on cell migration towards the same substrate. We tested the ability of various concentrations of lebeectin to inhibit integrin-dependent migration of HBMEC cells using haptotaxis assays towards fibronectin in modified Boyden chambers. Lebeectin readily inhibited HBMEC cell migration (Fig. 4A). Furthermore, lebeectin again exhibited a stronger inhibitory effect than did lebecetin (Fig. 4A and 4B). More precisely, the IC_{50} values for inhibition of HBMEC migration on fibronectin by lebeectin and lebecetin, are, respectively, 0.015 $\mu g/ml$ or 0.49 nM and 26.1 $\mu g/ml$ or 870 nM (Fig. 4B).

Lebectin blocks HBMEC tubulogenesis.

Cell adhesion and migration are integral parts of the angiogenic process and affect tubulogenesis. Since HBMEC tubulogenesis can be inhibited by specific anti- α V and anti- α 5 antibodies (data not shown), we hypothesized that lebectin-mediated impairment of HBMEC adhesion and migration could therefore affect tubulogenesis. HBMEC were treated with various concentrations of venom peptides and *in vitro* tubulogenesis assays were performed on Matrigel™. As shown in Figures 5A and 5B, lebectin strongly inhibited *in vitro* tubulogenesis. As previously shown for adhesion and migration assays, we observed stronger inhibition by lebectin (IC_{50} =0.012 μ g/ml or 0.39 nM) than by lebecetin (IC_{50} =2.45 μ g/ml or 80 nM) (Fig 5B).

Lebectin inhibits HBMEC proliferation.

In order to stabilize new vasculature during the angiogenic process, endothelial cells need to actively proliferate. Because lebectin strongly inhibited HBMEC angiogenesis *in vitro*, we investigated its effect on HBMEC proliferation. As expected, lebectin strongly inhibited HBMEC proliferation during a 5-day period (Fig. 6). Furthermore, lebectin again presented a greater inhibitory effect than did lebecetin. After five days, lebecetin inhibited HBMEC proliferation by 56% whereas lebectin inhibited HBMEC proliferation by 84% (Fig. 6). The apoptotic state of HBMEC cells in the -presence or absence of peptides was tested after 4 days using the PI/Annexin-5 assay. The results of this assay were negative (data not shown). This suggests that the lower number of cells observed in wells containing peptides is not due to greater cell death.

Lebectin displays antiangiogenic activity in vivo.

To pursue further the characterization of the antiangiogenic properties of lebectin, we performed two different *in vivo* angiogenesis assays. First, we looked at the effects of lebectin on angiogenesis, using a chicken CAM assay. 0.2 μ g of

leblectin strongly reduced vasculature development (Fig.7A). The total vessel length was reduced by 51% compared to the untreated conditions (Fig.7B). A strong reduction in the number of new capillaries and branching, without affecting mature blood vessels, was also observed (Fig.7A). This strongly suggests an antiangiogenic activity of leblectin *in vivo*. In accordance with our *in vitro* results, the lebecetin-mediated inhibition of angiogenesis was less than that seen with leblectin, with only 31% inhibition (Fig.7A and B), even though the concentration used was 50-fold higher.

In order to test the ability of leblectin to antagonize FGF-2-induced angiogenesis, we performed Matrigel™ plug assays in nude mice. To quantify the angiogenic index, we measured the levels of hemoglobin in the Matrigel™ plugs, as previously described (McMahon et al. 2001). FGF-2-containing Matrigel™ plugs appeared to be highly vascularized, compared to control plugs (Fig. 7C). More precisely, we measured a 7-fold higher amount of hemoglobin in the FGF-2-containing plugs (Fig. 7D). When leblectin was added to FGF-2-containing plugs, a strong decrease in vascularization was observed (Fig. 7C). When hemoglobin was measured, we observed a decrease of 43% in plug vascularization (Fig. 7D). This result clearly shows that leblectin can antagonize FGF-2-mediated angiogenesis *in vivo*. The effect of lebecetin on the FGF-2-induced angiogenic process was also tested. As shown by Figures 7C and D, no effect of lebecetin could be observed on the induced angiogenesis when lebecetin was present at the same concentration as was used for leblectin (5 µg). Consequently, we can conclude that leblectin, but not lebecetin, is able to block normal as well as induced angiogenesis *in vivo*.

DISCUSSION

Angiogenesis is implicated in numerous diseases such as rheumatoid arthritis, psoriasis and cancer (Folkman 1995a). Therefore, the characterization of new antiangiogenic agents is of considerable utility for the development of antiangiogenic therapies. Numerous active molecules have been discovered in the plant and animal kingdoms and studied for potential use in medicine. Most notably, it has been shown that several snake venom-derived peptides can affect the angiogenic process. Among these peptides, disintegrins have been widely characterized (McLane et al. 2004). For instance, it has been shown that disintegrins derived from the venoms of *Agkistrodon contortrix* (contortrostatin) (Golubkov et al. 2003), *Agkistrodon halys brevicaudus stejneger* (Adinbitor) (Wang et al. 2004) or *Vipera lebetina obtusa* (Obtustatin) (Marcinkiewicz et al. 2003) exhibit antiangiogenic activities. In the present study, we describe the antiangiogenic activity of a newly discovered CLP, lebectin, derived from *Macrovipera lebetina* snake venom.

Our data demonstrate that lebectin displays antiangiogenic activity at very low concentrations, both *in vitro* and *in vivo*. Lebectin, used at a concentration less than 1 nM, efficiently inhibits several key steps of the angiogenic process *in vitro*, such as endothelial cell adhesion, migration and tubulogenesis. Moreover, our results clearly demonstrate that low concentrations of lebectin efficiently block angiogenesis *in vivo* in two different assays. Thus, lebectin presents the best antiangiogenic efficacy yet described for snake venom-derived peptides (Golubkov et al. 2003; Marcinkiewicz et al. 2003).

We characterized integrin expression at the HBMEC cell surface by flow cytometry. In addition to the classical endothelial integrins ($\alpha V\beta 3$, $\alpha V\beta 5$ and $\alpha 5\beta 1$), we also demonstrated that $\alpha 6$ and $\beta 4$ are expressed in HBMEC. Because the $\beta 4$ subunit only interacts with the $\alpha 6$ integrin subunit, it seems likely that the

heterodimer $\alpha 6\beta 4$ is present at the HBMEC cell surface as a laminin receptor. Moreover, the high expression of $\alpha 2$, $\alpha 3$ and $\beta 1$ indicates the presence of $\alpha 2\beta 1$ and $\alpha 3\beta 1$, which could also participate in adhesion to laminin or to various collagens.

In a previous study, we hypothesized that lebeectin acts through the blocking of integrin-dependent processes involving the $\alpha 5\beta 1$ integrin (Sarray et al. 2004). Our present data confirm that low concentrations of lebeectin interacts with and impairs $\alpha 5\beta 1$ -mediated HBMEC adhesion to fibronectin, as well as $\alpha V\beta 3$ -mediated HBMEC adhesion to vitronectin. We clearly observed interaction between lebeectin and both $\alpha 5\beta 1$ and αV integrins. Despite the fact than lebeectin can interact with both $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins, it exhibits a higher affinity for $\alpha 5\beta 1$ integrin than for $\alpha V\beta 3$ integrin since lower concentrations of lebeectin are needed to inhibit the $\alpha 5\beta 1$ adhesive properties. This was confirmed by the finding that lebeectin readily inhibited cell adhesion to anti- $\alpha 5\beta 1$ antibodies while only partially affecting adhesion to anti- $\alpha V\beta 3$ antibodies.

The co-immunoprecipitation results clearly demonstrated that lebecetin interacts more strongly with αV integrin than does lebeectin. Contrary to this, lebeectin interacts more strongly with $\alpha 5$ integrin subunits than does lebecetin. Therefore, it would be expected that lebecetin would have a greater inhibitory effect on αV -mediated HBMEC cell adhesion on vitronectin than lebeectin, and that lebeectin would display a stronger inhibitory effect on $\alpha 5$ -mediated HBMEC adhesion on fibronectin. Our data clearly demonstrated that lebeectin actually blocked HBMEC cell adhesion and migration on vitronectin better than did lebecetin. The structural differences between these two CLPs might explain these differences since lebeectin presents a homodimeric structure whereas lebecetin displays a heterodimeric structure. In fact, the specific structure of lebecetin could favor interaction with specific integrins at the endothelial cell surface without affecting their functions. A full study of the structural domains of lebeectin and lebecetin, which interact with integrins at the cell surface, could thus explain the different interaction potentials and anti-angiogenic activities of

these two CLPs. The interaction between lelectin and integrins still remains to be clarified and its characterization is currently in progress in our lab.

$\alpha 5 \beta 1$ has already been reported to play a critical role in the development of new blood vessels. One study demonstrated that $\alpha 5$ -null mouse embryos displayed a marked decrease in the complexity of the vasculature than was seen in wild-type embryos (Francis et al. 2002). The heterodimer $\alpha 5 \beta 1$ has also been shown to be expressed in growing vessels but its expression disappears in mature vessels (Milner and Campbell 2002). Recent work revealed the reexpression of $\alpha 5 \beta 1$ integrin in tumor vessels, which was not present in stable blood vessels in adult (Kim et al. 2000b). Because low concentrations of lelectin inhibit angiogenesis through the blockade of the $\alpha 5 \beta 1$ integrin, lelectin should thus preferentially affect endothelial cells in growing vessels, which actively participate in the tumoral angiogenic process. Our results showed that lelectin does not affect stable, established blood vessels on CAMs but strongly reduces the number of new capillaries and vascular branching. Moreover, lelectin does not affect the viability of stable confluent endothelial cells *in vitro* (data not shown). Consequently, low concentrations of lelectin may specifically target activated endothelial cells and block neoangiogenesis, without affecting stable vasculature.

Previous results demonstrated the antitumoral potential of lelectin (Sarray et al. 2004). Remarkably, the concentrations of lelectin used to block tumor cell adhesion (over 10 $\mu\text{g/ml}$) are about 100-fold higher than the concentrations needed to display antiangiogenic activity ($\approx 0.1 \mu\text{g/ml}$). Thus, endothelial cells seem to be more sensitive to lelectin than are various tumor cells, suggesting that lelectin is a better antiangiogenic agent than an antitumoral one. This may be explained by the higher expression of $\alpha 5 \beta 1$ and $\alpha V \beta 3$ integrins in endothelial cells compared to tumoral cells. Lelectin could thus be very useful for the specific treatment of brain tumors. Conventional therapies, such as chemotherapy and radiotherapy, fail to strongly

affect brain tumor cells due to the presence of the blood brain barrier and the intrinsic multidrug-resistant phenotype of these type of tumors (Graham and Cloughesy 2004; Matsutani 2004). Antiangiogenic therapies have been presented as a new hope for the treatment of brain tumors (Purow and Fine 2004b, 2004a). According to our present results, we can hypothesize that lebeclin could be used with high efficiency for the inhibition of the brain angiogenic process. Therefore, it is conceivable that lebeclin could open new avenues for the treatment of brain tumors.

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FIGURE LEGENDS

Figure 1: **Basal motility properties of HBMEC.** (A) Cells (5×10^4) were seeded on 96-well plates previously coated with various concentrations of different, purified ECM proteins and allowed to adhere for 2 hours at 37°C. Cells which adhered were fixed, stained and absorbance at 600 nm was measured. (B) Haptotaxis assays were performed as described in the Materials and Methods section. The lower faces of the Transwells were previously coated with either type I collagen, fibronectin, laminin-1 or vitronectin. Cells that migrated to the underside were fixed, stained and counted. Bar graph represents a mean quantification $\pm$ SEM of at least three independent experiments.

Figure 2: **Expression of integrin subunits at the HBMEC surface.** Flow cytometric analysis was performed using various antibodies directed against specific integrin subunits as described in the Materials and Methods section. Analysis was repeated three times.

Figure 3: **Lebectin inhibits HBMEC adhesion by blocking $\alpha 5\beta 1$ and $\alpha V\beta 3$ adhesive functions.** (A) HBMEC (10^6 cells/ml) were treated with various concentrations of lebectin (open circles) or lebecetin (closed circles) for 30 min at room temperature. 5×10^4 cells were then allowed to adhere to fibronectin (FN) or vitronectin (VN) as described in Figure 1. (B) HBMEC (10^6 cells/ml) were treated (white bars), or not (black bars), with 10 μ g/ml of lebectin for 30 min. at room temperature and allowed to adhere on various anti-integrin antibodies. Adhesive cells were fixed, stained and absorbance at 600 nm was measured. ★ indicates statistically significant differences ($p < 0.05$) compared with the control assay using Student's *t*-test. (C) HBMEC (10^6 cells/ml) were incubated with 50 μ g/ml of lebectin or lebecetin for 5 min, lysed and the peptides were co-immunoprecipitated using an anti-lebecetin antiserum. $\alpha 5$ and αV integrins were detected by Western blot.

Figure 4: Lebectin inhibits HBMEC migration. HBMEC (10^6 cells/ml) were treated with various concentrations of lebectin (open circles) or lebecetin (closed circles) for 30 min at room temperature. HBMEC (5×10^4 cells) were seeded on top of fibronectin-coated Transwells and allowed to migrate for 2 hours at 37°C . (A) Representative data are shown. (B) Cells that had migrated were fixed, stained and counted as described in the Materials and Methods section. Graph represents a mean quantification $\pm$ SEM of at least three independent experiments. Results are expressed as percentage of control cells.

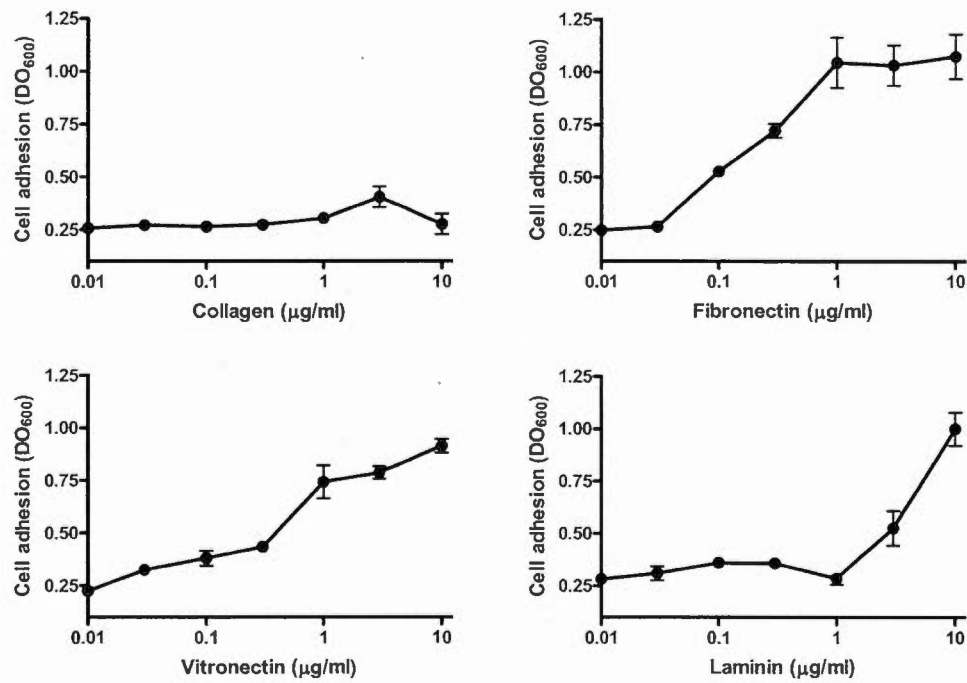
Figure 5: Lebectin strongly inhibits HBMEC tubulogenesis on Matrigel. HBMEC (10^6 cells/ml) were treated with various concentrations of lebectin (open circles) or lebecetin (closed circles) for 30 min at room temperature. Morphogenic differentiation assays were performed as described in the Materials and Methods section. Cells (5×10^4) were added to the MatrigelTM-coated wells in serum-free medium and allowed to form capillary-like structures for 18 hours at 37°C . (A) A representative tubulogenesis assay is shown after treatment of HBMEC with varying concentrations of lebectin or lebecetin. (B) The capillary-like structures formed in the gel were quantified and a mean quantification is presented. Graphs represent a mean quantification $\pm$ SEM of at least three independent experiments.

Figure 6: Lebectin reduces HBMEC proliferation. HBMEC (2000 cells/well) were allowed to grow in 96-well plates for 5 days in 10% serum-containing medium with various lebectin (open circles) or lebecetin (closed circles) concentrations. As a control (open squares), cells were incubated with 10% serum-containing medium alone. Each day, three wells were washed twice with PBS, fixed and stained. Absorbance at 600 nm was then measured. ★ indicates statistically significant differences ($p < 0.05$) compared with the control assay using Student's *t*-test.

Figure 7: Lebectin blocks *in vivo* angiogenesis. Antiangiogenic properties of lebectin were monitored *in vivo* by CAM assays and Matrigel™ plug assays in nude mice. (A) Chicken CAM were untreated (control) or treated with lebectin (0.2 µg) or lebecetin (10 µg) and a quantitative measurement of vessel density was performed by counting vessel length per embryo. (B) Crl:CD-1®-nuBR mice were injected subcutaneously with 0.5 mL of Matrigel™ alone or with Matrigel™ containing FGF-2 (125 ng) with or without lebectin or lebecetin (5 µg). After 7 days, the mice were euthanized and the Matrigel™ plugs excised. Representative Matrigel™ plugs from each of the different conditions were photographed. Neovessel formation was quantified by measurement of hemoglobin in the Matrigel™. Two mice were used per group and the experiment was repeated twice. The data are presented as mean +/- SEM. Statistically significant differences, as compared to control FGF-2 conditions (as well as between the two control conditions), are indicated by ★ for $p < 0.05$; ★★ for $p < 0.001$ (Student's *t*-test).

Figure 1: Basal motility properties of HBMEC

A)



B)

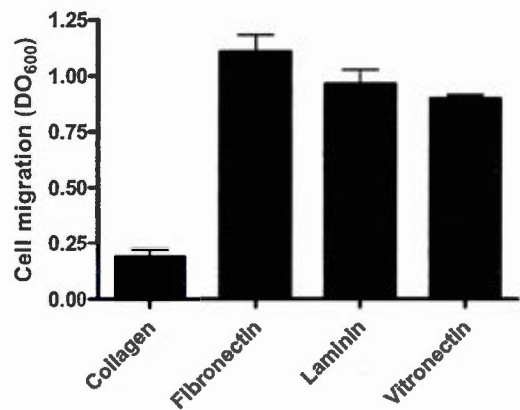


Figure 2: Expression of several integrin subunits at the HBMEC surface

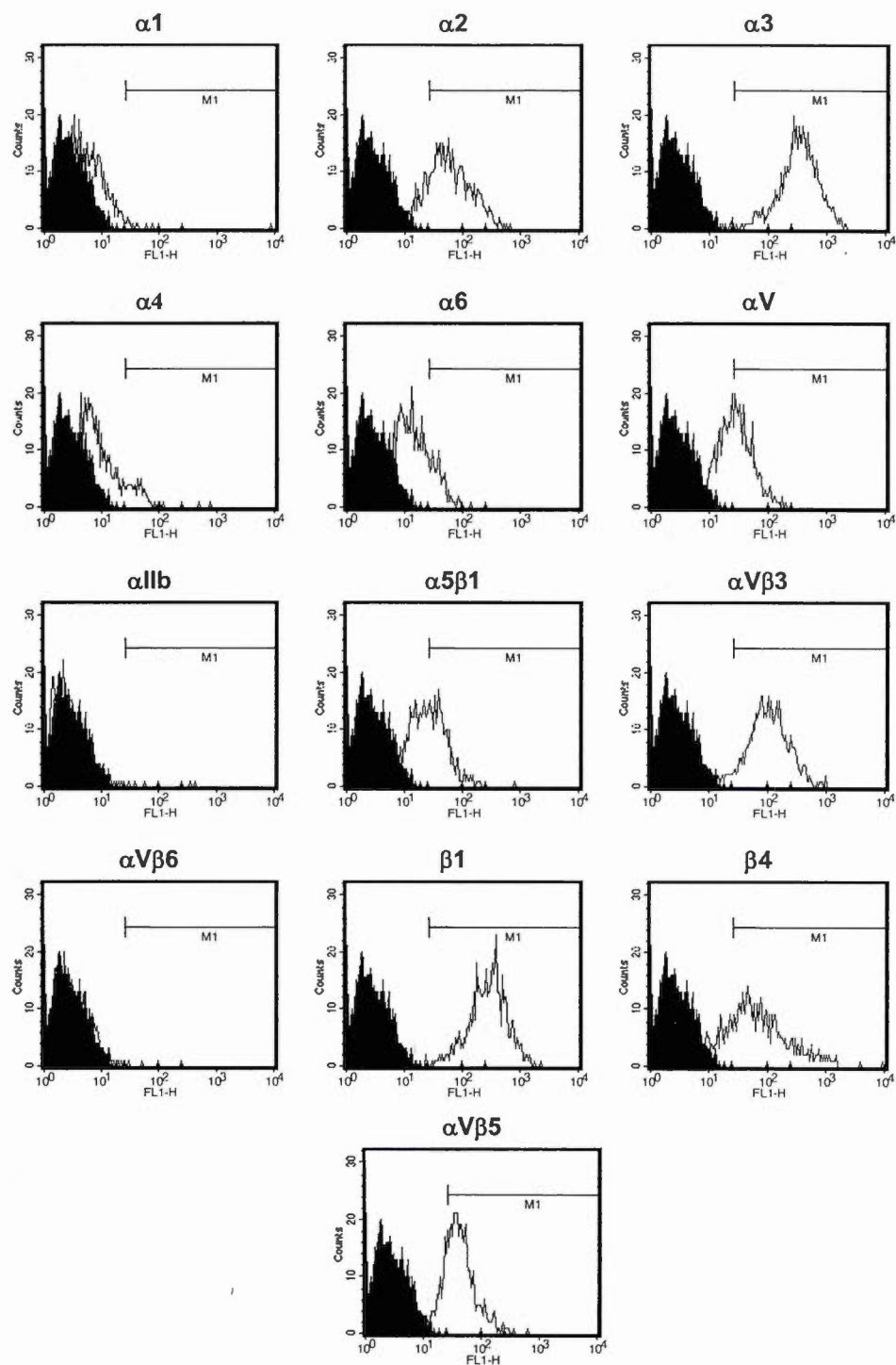
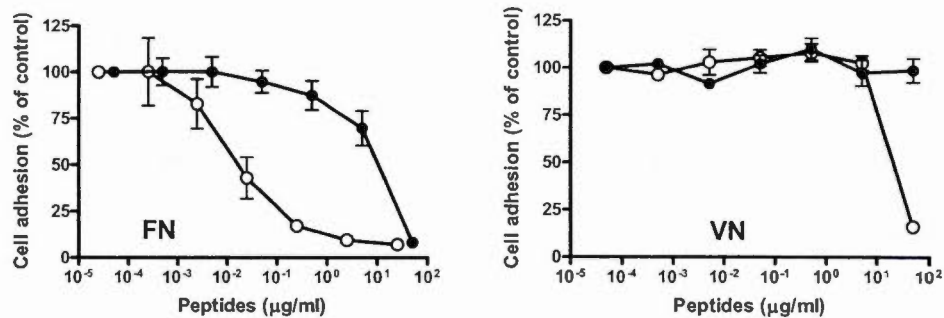
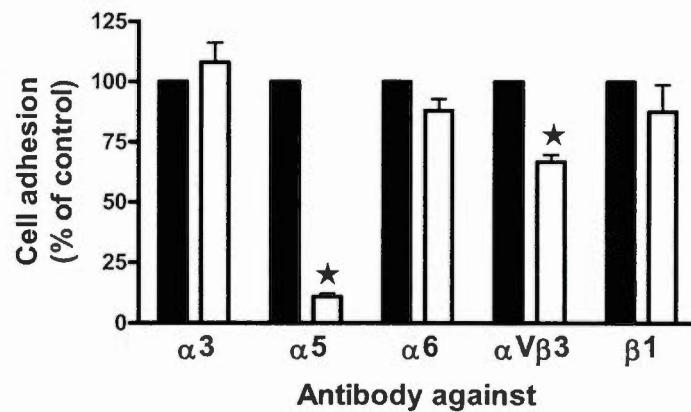


Figure 3: Lebectin inhibits HBMEC adhesion by blocking $\alpha 5\beta 1$ and $\alpha V\beta 3$ adhesive functions

A)



B)



C)

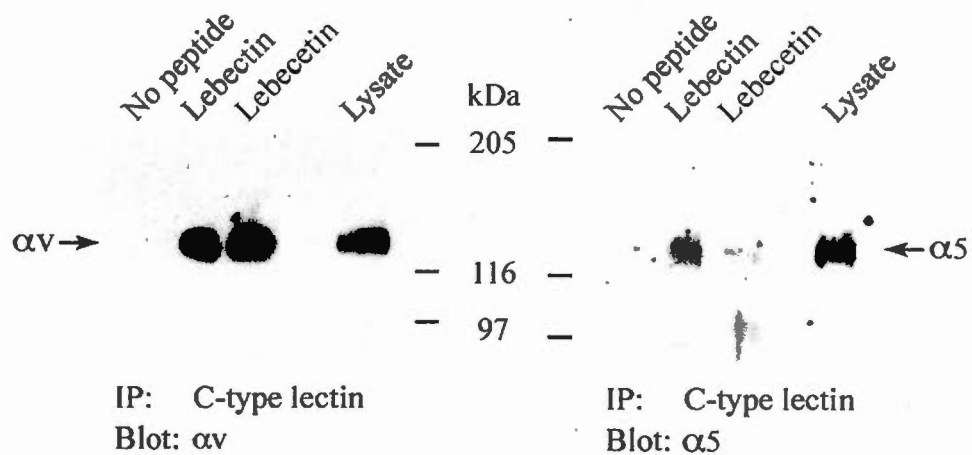
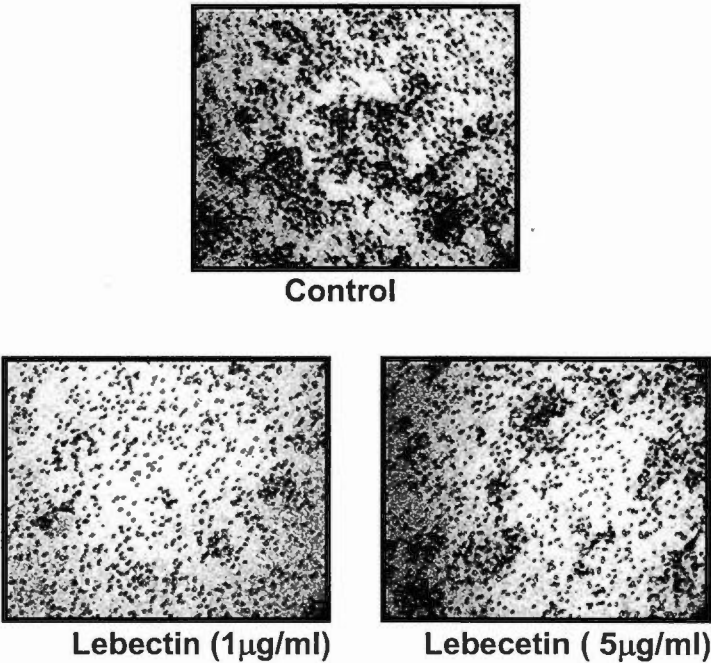


Figure 4: Lebectin inhibits HBMEC migration

A)



B)

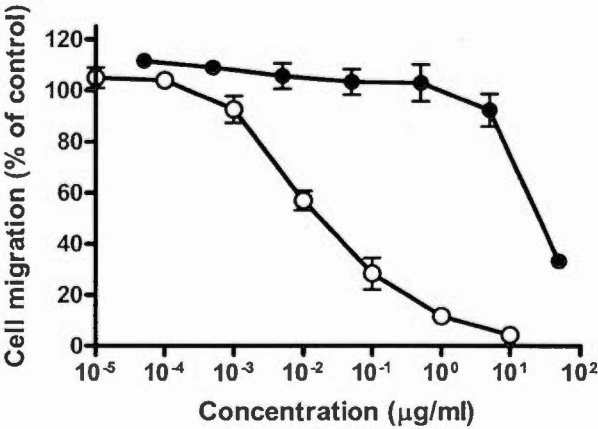
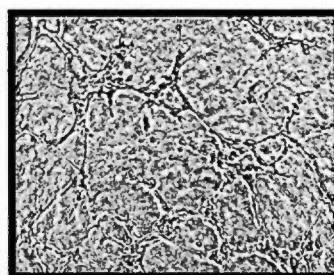
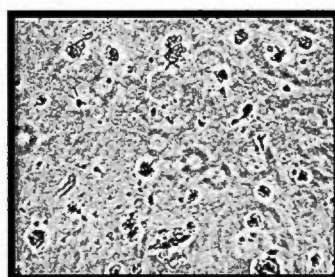


Figure 5: Lebectin firmly inhibits HBMEC tubulogenesis on Matrigel

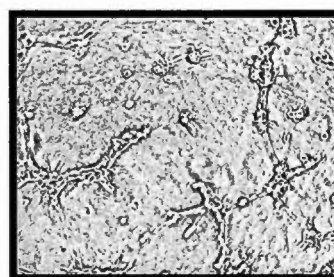
A)



Control



Lebectin (1 µg/ml)



Lebectin (5 µg/ml)

B)

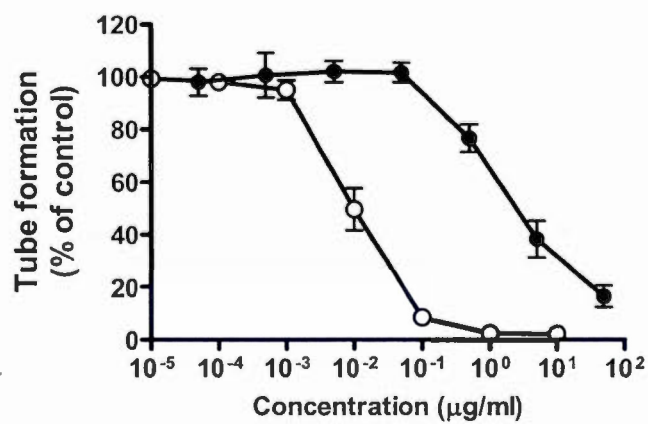


Figure 6: Lebectin reduces HBMEC proliferation

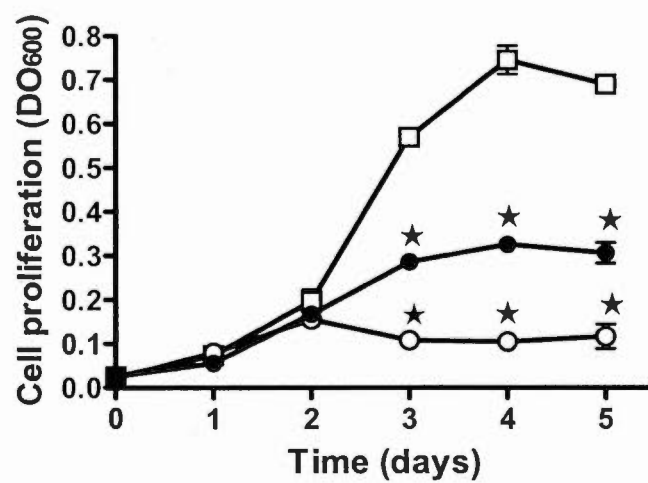
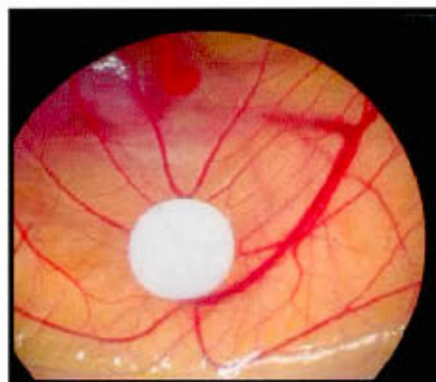
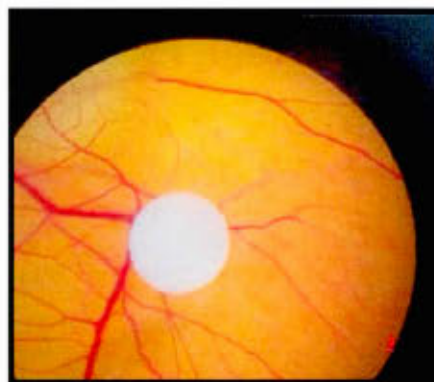


Figure 7: Lebectin blocks *in vivo* angiogenesis

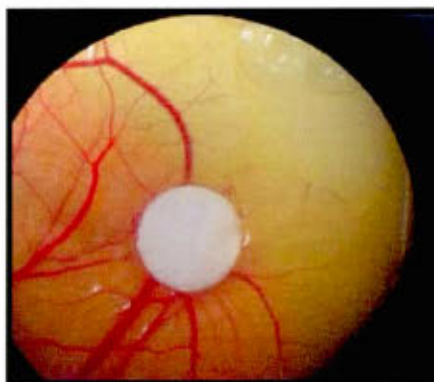
A)



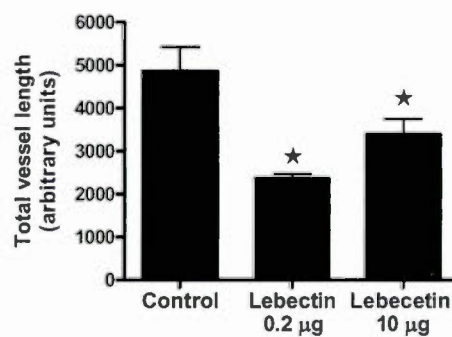
Control



Lebectin (0.2μg)



Lebecetin (10μg)



B)



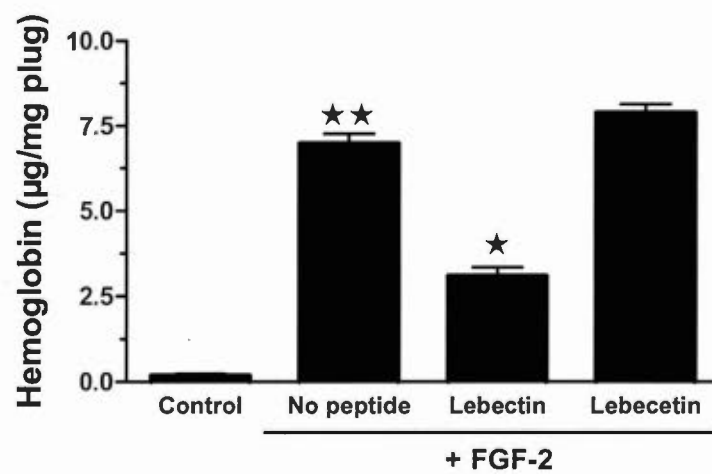
Control

No peptide

Lebectin

Lebectin

+ FGF-2



Article 3 : Inhibition of angiogenic properties of brain endothelial cells by platelet-derived sphingosine-1-phosphate

Anthony Pilorget^{1,4}, Borhane Annabi², Fatiha Bouzeghrane³, Jacques Marvaldi⁴, José Luis⁴ and Richard Béliveau^{1*}

¹*Laboratoire de Médecine Moléculaire, Centre de Cancérologie Charles-Bruneau, Hôpital Sainte-Justine-UQAM, Montreal, Quebec, Canada,* ²*Laboratoire d'Oncologie Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal, Montreal, Quebec, Canada,* ³*Laboratory of the Cell Biology of Hypertension, Clinical Research Institute of Montreal, University of Montreal, Montreal, Quebec, Canada,* and ⁴*CNRS FRE 2737, Faculté de Pharmacie, 27, bd Jean Moulin, 13385 Marseille, Cedex 5, France*

* To whom correspondence and reprint requests should be directed

Laboratoire de Médecine Moléculaire

Université du Québec à Montréal

C.P. 8888, Succursale Centre-ville

Montréal, Québec

CANADA H3C 3P8

Phone : (514) 987-3000 ext 8551

Fax : (514) 987-0246

E-mail : oncomol@nobel.si.uqam.ca

The abbreviations used are: EC, endothelial cells; ECM, extracellular matrix; LPA, lysophosphatidic acid; LPL, lysophospholipids; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1-MMP; RBE4, Rat Brain Endothelial cell; S1P, sphingosine-1-phosphate; S1P-1, sphingosine-1-phosphate receptor-1.

CONTRIBUTION

Ce troisième article a permis de mieux comprendre le rôle complexe joué par la sphingosine-1-phosphate (S1P) dans le processus angiogénique cérébral. Nos résultats mettent en évidence un rôle antiangiogénique de la S1P dans les cellules endothéliales cérébrales de rat RBE4. Nous avons aussi démontré que l'absence de MT1-MMP dans les cellules RBE4 était à l'origine de l'effet antiangiogénique de la S1P. Cet article a permis de démontrer l'effet histospécifique de la S1P sur l'endothélium vasculaire ainsi que de confirmer le caractère phénotypique unique de l'endothélium vasculaire cérébral.

J'ai réalisé les expériences, en collaboration avec le Dr Fatiha Bouzeghrane, sous la supervision directe du Dr Borhane Annabi et de mes deux co-directeurs, le Dr Richard Béliveau et le Pr José Luis. J'ai rédigé cet article et sa relecture a été faite par le Pr José Luis, le Dr Richard Béliveau et le Dr Denis Gingras. Cet article a été publié dans la revue scientifique *The Journal of Cerebral Blood Flow and Metabolism* en septembre 2005.

RÉSUMÉ EN FRANÇAIS

La sphingosine-1-phosphate (S1P) est un sphingolipide sécrété par les plaquettes activées, stimulant les propriétés angiogéniques des cellules endothéliales (CE) de diverses origines tissulaires. La régulation paracrine de l'angiogenèse cérébrale par les facteurs dérivés des plaquettes est cependant peu étudiée. Dans la présente étude, nous avons étudié le rôle de la S1P dans la régulation de la migration et de la tubulogenèse des CE cérébrales, en utilisant la lignée cellulaire endothéliale cérébrale de rat RBE4. Nous démontrons que la S1P inhibe la migration et la tubulogenèse des CE cérébrales, alors que la S1P stimule les propriétés angiogéniques des CE non cérébrales. La surexpression du récepteur S1P-1 dans les cellules RBE4 potentialise l'effet antiangiogénique de la S1P. Nos résultats suggèrent aussi que l'absence, dans les CE cérébrales, de MT1-MMP, une métalloprotéase de matrice membranaire coopérant avec la S1P pour induire la tubulogenèse endothéliale, peut expliquer les effets antiangiogéniques de la S1P. Globalement, nos résultats démontrent que la S1P a un rôle antiangiogénique et histospécifique au niveau de l'endothélium cérébral. La S1P pourrait ainsi aider à stabiliser le lit vasculaire cérébral et avoir un impact majeur sur la mise en place et la régulation de la vascularisation cérébrale normale.

ABSTRACT

The platelet-derived lysophospholipid sphingosine-1-phosphate (S1P) is present in blood plasma and is one of the most potent growth factors displaying proangiogenic activity towards endothelial cells (EC) derived from various tissues. The paracrine regulation of brain angiogenesis by platelet-derived growth factors is, however, poorly understood. In the present study, we assessed the role of S1P on brain EC migration and tubulogenesis, using rat brain-derived (RBE4) EC as an *in vitro* model. We show that S1P inhibits brain EC migration and tubulogenesis, while it displays proangiogenic activity towards non-cerebral EC. Overexpression of the S1P receptor S1P-1 in RBE4 cells potentiated all of the S1P-mediated events. We also demonstrate that the lack of expression of MT1-MMP, a membrane-bound matrix metalloproteinase that is thought to cooperate with S1P in tubulogenic processes, may explain the antiangiogenic activity of S1P on brain vasculature. Altogether, our results support the hypothesis of a tissue-specific, antiangiogenic role of S1P in the brain, which may help to stabilize the cerebral vasculature and thus have crucial impact on the setting and regulation of normal brain vascularization.

INTRODUCTION

Brain vascularization is performed through two different mechanisms, vasculogenesis (Mato et al. 1989) and angiogenesis (Plate 1999). The latter is a physiological process that is fundamental to normal healing, reproduction and embryonic development (Zadeh and Guha 2003). However, angiogenic processes have also been implicated in the pathogenesis of a wide variety of disorders, including primary and metastatic brain tumors, aneurysms, arteriovenous malformations and cavernous malformations (Harrigan 2003). Angiogenesis is initiated by the release of proteases from activated endothelial cells (EC), leading to the degradation of the basement membrane, migration into the interstitial space, with subsequent EC proliferation and differentiation into mature blood vessels. Each of these processes is tightly regulated through the complex interplay of endogenous factors that promote and inhibit angiogenesis (Folkman 2003). Among them, VEGF (*vascular endothelial growth factor*) and bFGF (*basic fibroblast growth factor*) have been shown to promote strong proangiogenic activity in EC (Distler et al. 2003).

The specific process of brain angiogenesis has been shown to be sustained by several growth factors, such as VEGF, bFGF, PDGF (*Platelet-derived Growth Factor*) and TGF- β (*Transforming Growth Factor- β*) (Risau et al. 1998; Harrigan 2003; Niimi 2003). Numerous bioactive lipids have also been identified as potent angiogenesis modulators, including platelet-derived lysophospholipids (English et al. 2002). The prominent members of the family of lysophospholipids (LPL) are the structurally related molecules sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) that are secreted by activated platelets (Xu et al. 2003). Several studies have documented the effect of LPL on the proliferation properties of cell types such as EC or fibroblasts (Panetti 2002; Rosen and Goetzl 2005). LPL act through homologous G protein-coupled receptors (GPCRs), known as the Edg (*Endothelial Differentiation Genes*) receptors, but recently named S1PR and LPAR (An et al.

1998; Rosen and Goetzl 2005). S1P preferentially activates EDG-1/S1P-1, EDG-5/S1P-2, EDG-3/S1P-3, EDG-6/S1P-4, and EDG-8/S1P-5, whereas LPA activates EDG-2/LPA-1, EDG-4/LPA-2, and EDG-7/LPA-3 (Chun et al. 2002; Anliker and Chun 2004). Moreover, S1P may also specifically act as an intracellular second messenger (Spiegel and Milstien 2003).

Platelet-derived lysophospholipid S1P is a lysosphingophospholipid present in blood plasma, largely in albumin- and lipoprotein-bound forms (English et al. 2000; Yatomi et al. 2000). The S1P serum concentration ranges from about 0.2 to 1 μ M (Okajima 2002). This bioactive sphingolipid metabolite is generated through the phosphorylation of sphingosine by sphingosine kinase (Ancellin et al. 2002) and has been shown to elicit a great variety of responses, including stimulation of cell proliferation, survival, and cytoskeletal reorganization (Maceyka et al. 2002; Spiegel and Milstien 2003). One of the most widely studied functions of S1P is the regulation of the cell migration process and its role in angiogenesis, as it stimulates migration and promotes tubulogenesis in EC (English et al. 2000; Panetti 2002).

However, little is known about the role of S1P in regulating the angiogenic process in brain. Because EC migration and tubulogenesis represent crucial steps of angiogenesis, we investigated how the motility of brain-derived EC can be modulated by S1P. In this work, we demonstrate that S1P acts as a specific inhibitor of brain EC migration and tubulogenesis *in vitro*. Moreover, we show that the S1P-1 receptor specifically mediates the S1P antiangiogenic effects on brain endothelium. We also demonstrate that MT1-MMP, a membrane-bound matrix metalloproteinase that is highly involved in the angiogenic process, is not expressed in brain EC. Finally, we establish that the absence of MT1-MMP in brain EC, which usual function is to cooperate with S1P to trigger tubulogenic processes in non-cerebral EC (Langlois et al, 2004), can explain the antiangiogenic role of S1P observed *in vitro*.

MATERIAL AND METHODS

Antibodies and chemicals : TriZOL reagent, trypsin, geneticin (G418), penicillin and streptomycin were from Invitrogen (Burlington, ON). Fibronectin was from Roche (Mississauga, ON). Type-1 collagen was extracted from rat tail tendon by standard method (Silver and Trelstad 1980). Vitronectin was prepared as previously described (Yatohgo et al. 1988). Recombinant PDGF, VEGF, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were from R&D Systems (Minneapolis, MN). S1P, LPA, concanavalin A (ConA), cytochalasin D (CytoD), EDTA, agarose, gentamycin, laminin-1, sodium dodecyl sulfate (SDS), trypsin for EC and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). bFGF was from Upstate Biotechnology (Lake Placid, NY). Epithelial growth factor (EGF) and Matrigel™ were from BD Biosciences Labware (Bedford, MA). Anti human MT1-MMP polyclonal antibody (raised against the hinge region), anti-TIMP-2 polyclonal antibody, and the enhanced chemiluminescence-Western blot kit were from Chemicon International (Temecula, CA.).

Cells and culture media : Dulbecco's Modified Eagle's medium (DMEM), MEM, α -MEM medium, MDCB 131 medium, Ham's F12 Nutrient Mixture and L-Glutamine were purchased from GIBCO BRL (Burlington, ON). Fetal bovine serum (FBS) and fetal calf serum (FCS) were from HyClone Laboratories (Logan, UT). The CNS-1 murine glioma cells, kindly provided by Dr W. F. Hickey, were cultured in RPMI 1640 supplemented with 10% FCS. Human umbilical vein EC (HUVEC) were purchased from ATCC and were maintained in EGM-2 Bulletkit medium (Clonetics, San Diego, CA). Human dermal microvascular EC (HMEC-1) were kindly provided by the Centers for Disease Control and Prevention (Atlanta, TX). HMEC-1 cells were grown in MDCB 131 medium and used up to passage 20. Bovine aortic EC (BAEC) were from Clonetics and cultured in low glucose DMEM containing 10% heat-

inactivated FCS (MediCorp, Montreal, QC), 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 1 ng/ml bFGF and used up to passage 12. Bovine brain capillary EC (BBCEC) were isolated and maintained as previously described (Cecchelli et al. 1999). Because of their bovine origin, BBCEC were not used for immunodetection experiments. RBE4 immortalized rat brain microvessel EC were gift of Dr Françoise Roux. GP8.39 immortalized rat brain microvessel EC were kindly provided by Dr John Greenwood. Due to their relatively poor transfection efficiency, GP8.39 were not used for cDNA transfection experiments. RBE4 and GP8.39 cells were plated on type I collagen-coated plates (200 μ g/ml) and maintained in α MEM/Ham's F12 (1:1 v/v) containing 10% heat-inactivated FCS, 30 mg/ml geneticin, 50 mg/ml gentamycin and 100 ng/ml bFGF. All cells were incubated at 37°C, air/CO₂ (95%/5%).

Morphogenic differentiation assay: Matrigel™ was plated in 96-well plates and allowed to gel for 2 hours at 37°C prior to cell seeding. RBE4 cells (5×10^4) were added atop the Matrigel™ in serum-free media in the presence or absence of S1P and incubated at 37°C for 18 hours. Capillary-like structures were analyzed microscopically and photographs (50X) taken with a Retiga 1300 camera connected to a Nikon Eclipse TE2000-U microscope. The capillary-like structures formed in the gel were quantified by analysis of digitized images to determine the segment length of the capillary-like network, using the Northern Eclipse analysis software (Empix Imaging inc., Mississauga, ON). Results are expressed as percentage of control cells, and are the means of at least three independent experiments. Error bars represent SD of values.

cDNA constructs and cell transfection: A plasmid encoding S1P-1-GFP chimera was generously provided by Dr Timothy M. Palmer. The cloning of human MT1-MMP cDNA has been described previously (Demeule et al. 2002b). RBE4 cells were transiently transfected with cDNA plasmids by using the non-liposomal

formulation FUGENE-6 transfection reagent (Boehringer Mannheim, Laval, QC). Transfection efficiency was confirmed by western blotting. All experiments involving transfected cells were performed 36 hours following transfection. Mock transfections of RBE4 cells with pcDNA (3.1+) expression vector alone were used as controls.

Cell adhesion assays: Adhesion assays were performed as previously described (Rigot et al. 1998). Briefly, wells were coated with purified extracellular matrix (ECM) protein solutions for 2 hours at 37°C, then blocked with a solution of PBS/BSA 0.5%. Matrix concentrations used were as follow: Type-I collagen, fibronectin, laminin-1, vitronectin, elastin (10µg/ml), fibrin (0.25 % w/v in NaOH 1N), hyaluronan (1mg/ml) and casein (0.25% w/v in PBS). Cells were then harvested in single cell suspension by brief trypsinization, added to wells precoated with purified ECM proteins, and allowed to adhere to the substrata for 2 hours at 37°C. After washing, adherent cells were stained with a solution of 0.1% crystal violet/MetOH 20% and lysed with 1% SDS. Absorbance was then measured at 600 nm. All assays were performed in triplicate and data are the means of at least three independent experiments. Error bars represent SD of values.

Migration assays: To assess the EC migratory potential, the lower face of Transwells (8µm pore size; Costar, Acton, MA) was precoated with type-1 collagen, laminin-1, vitronectin or fibronectin, at a concentration of 10 µg/ml, for 2 hours at 37°C. The Transwells were then assembled in a 24-well plate (Fisher Scientific Ltd, Nepean, ON), the lower chambers were filled with 600 µl of serum-free medium, and 100 µl of cells (10^6 cells/ml) were inoculated onto the upper chamber of each Transwell. Various concentrations of growth factors in serum-free medium were added to the lower chambers of the Transwells. The plate was then placed at 37°C in 5% CO₂/95% air for 1 hour. Cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet/20% (v/v) MeOH. All assays were performed in triplicate. The migration was quantified using computer-assisted

imaging and data were analyzed using the Northern Eclipse software. Migration data are expressed as a mean value of, at least, three independent experiments. Error bars represent SD of values.

Isolation of EC fractions from brain tissue: The isolation of EC from normal male Lewis rat (Charles River, QC, Canada) brains has been performed as previously described (Regina et al, 2003). All animal experiments were evaluated and approved by the Institutional Comity for Good Animal Practices (UQAM, Montréal, QC, Canada). Magnetic cell sorting (MACS) was performed using microbeads, a MidiMacs separation unit and positive selection MACS columns from Miltenyi Biotec (Auburn, CA, USA). Mouse anti-PECAM-1 antibody that was linked to microbeads was from Cedarlane Laboratories (Hornby, ON, Canada).

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis: Total RNA was extracted from cells using the TriZOL Reagent. RT-PCR reactions were performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq Kit from Invitrogen (Burlington, ON). PCR conditions have been optimized so that the gene products were examined at the exponential phase of the amplification. PCR products were resolved on 2% (w/v) agarose gels containing 1 µg/ml ethidium bromide.

Immunoblotting procedures: Cells were homogenized in lysis buffer. Twenty µg of protein from control or transfected cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in

TBST containing 3% BSA, followed by a 1 hour incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/5,000 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence.

Gelatin zymography: Gelatinolytic activity in culture media from monolayer cultures was detected by gelatin zymography as previously described (Beaulieu et al. 1997). Briefly, an aliquot (20 μ l) of the culture medium was subjected to SDS/PAGE using a 7.5% (w/v) acrylamide gel containing 0.1 mg/ml gelatin. The gels were then twice incubated for 30 min at room temperature (25°C) in 2.5% (v/v) Triton X-100 to remove SDS and rinsed five times in double-distilled water. The gels were further incubated at 37°C for 20 hours in 20 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, 50 mM Tris/HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant Blue R-250, and destained in 10% (v/v) acetic acid, 30% (v/v) methanol in H₂O. Gelatinolytic activity was detected as unstained bands on a blue background and was quantified by densitometric measurement. All experiments were carried out with cells that had been serum-deprived by an 18 hours incubation.

Treatment with antisense oligonucleotides: Antisense phosphorothioate oligodeoxyribonucleotides (PTO) against S1P-1, S1P-3 and their respective scrambled controls were custom synthesized based on published sequences (Lee et al. 1999; Liu et al. 2001). The sequences of the oligonucleotides used are the following: S1P-1 antisense, 5'-GAC GCT GGT GGG CCC CAT-3'; scrambled S1P-1, 5'-TGA TCC TTG GCG GGG CCG-3'; S1P-3 antisense, 5'-CGG GAG GGC AGT TGC CAT-3'; scrambled S1P-3, 5'-ATC CGT CAA GCG GGG GTG-3'. To reduce S1P-1 and S1P-3 gene expression, RBE4 cells were grown to approximately 60% of confluence and incubated with 200 nM of either the scrambled controls or the S1P-1

or S1P-3 antisense oligonucleotides using Fugene-6 transfection reagent. After 72 hours of incubation, cells were used for migration and morphogenic assays.

Semi-quantitative real-time PCR analysis: Real-time PCR analysis was performed in a 2-step manner. cDNA synthesis and real-time detection were carried out in a Perkin Elmer Gene Amp PCR system and with a Mx4000 system (Stratagene, La Jolla, CA) respectively. Total RNA was extracted as described above. S1P-1 mRNA (5 µg) was reverse transcribed in a final volume of 20 µL containing 1 µL of 10 mmol/L dNTP, 4 µL BRL 5x buffer, 0.5 µL oligo-(dT)₁₂₋₁₈ primer (0.5 µg/µL, Invitrogen), 2 µL of 0.1 mol/L DTT and 1 µL of 200 U/µL M-MLV reverse transcriptase (Invitrogen) for 1 h at 37°C. The reaction was stopped by heating at 95°C for 5 min. The QuantiTect SYBR Green PCR kit (Qiagen, Mississauga, ON) was used in subsequent PCR assays according to the manufacturer's protocol. Gene expression was compared by the determination of target quantities in experimental samples relative to a calibrator sample (control), and expression levels were corrected for a normalizer gene, 16S rRNA. Single-strand cDNA (2 µL) was used for real-time PCR to amplify a 546-bp fragment of S1P-1 cDNA with the complementary sense primer 5'-GTCCGGCATTACAACACTACAC-3' and the antisense primer 5'-TATAGTGCTTGTGGTAGAGC-3'. For a 107-bp fragment of the housekeeping gene ribosomal protein S16 cDNA, the antisense primer was 5'-GCTACCAGGGCCTTTGAGATG-3', the sense primer, 5'-AGGAGCGATTTGCTGGTGTGG-3'. Thus, 2 µL of cDNA was amplified with specific primers at a final concentration of 1 µmol/L. All real-time RT-PCR experiments were performed in duplicate and were conducted with an initial denaturing interval (95°C, 15 min) and then with 40 sequence cycles for S1P-1 and S16: 94°C (30 s), 55°C (45 s), and 72°C (30 s). Melting-curve analysis was also performed to control for the absence of primer dimers.

RESULTS

Dependency of RBE4 cell adhesion and migration on substrate.

Although RBE4 cells have been extensively used as an *in vitro* model for the blood-brain barrier (Lagrange et al. 1999; Mroczkowska et al. 2000; Chishty et al. 2002), relatively little data is available concerning their motility properties. Since we were interested in several distinct processes involved in angiogenesis, we first characterized the adhesive and migratory properties of these cells on various purified ECM proteins. We determined that RBE4 cells exhibited approximately 7-fold higher adhesion to fibronectin than on any other matrix (Fig. 1A). We used modified Boyden chambers to assess the ability of RBE4 to migrate towards various purified ECM proteins including type I collagen, laminin, vitronectin and fibronectin. In accordance with the adhesion test results, RBE4 cell migration was significantly higher towards fibronectin (10-fold higher) than towards any other ECM protein tested (Fig. 1B). All subsequent migration experiments were consequently performed on fibronectin-coated filters.

Modulation of RBE4 cell migration by various growth factors.

Because brain EC are exposed to the effects of various growth factors *in vivo*, we assessed whether some of these factors can affect RBE4 cell migration. RBE4 were thus seeded on fibronectin-coated Transwells, and allowed to migrate for 1 hour towards serum-free medium supplemented with either serum (FBS 10%), S1P, PDGF, VEGF, TNF α , bFGF or GM-CSF. RBE4 displayed a strong chemotactic migration towards serum, VEGF, bFGF, PDGF and GM-CSF (Fig. 2). In contrast, S1P inhibited RBE4 cell migration by approximately 50%. Because this result is the first to report a potent role for S1P on the inhibition of brain EC migration, we decided to further characterize the effect of S1P on RBE4 cell migration and on tubulogenesis, another crucial event in brain angiogenesis.

Modulation of angiogenic properties of RBE4 cells by S1P

Varying S1P concentrations were used to assess RBE4 cell migration and tubulogenesis. As shown in Figure 3A, S1P inhibits RBE4 cell migration in a dose-dependent manner. RBE4 cells were also plated on Matrigel™ with varying S1P concentrations and incubated for 18 hours. S1P was also found to inhibit RBE4 *in vitro* tubulogenesis in a dose-dependent manner (Fig.3B). The unexpected results obtained with RBE4 cells were the opposite of those reported for non-cerebral EC. We thus investigated whether the precise tissue origin of EC could determine the different effects of S1P on angiogenesis.

Effect of S1P on the migration of cerebral and non-cerebral EC.

We tested the chemotactic activity of S1P on the migratory potential of various EC lines: HUVEC (Umbilical Vein), HMEC-1 (Dermal Microvascular), BAEC (Aortic), RBE4 (Cerebral), GP8.39 (Cerebral) and BBCEC (Cerebral). As shown in Figure 4, S1P stimulated the migration of all EC lines, which originated and were isolated from outside the brain. However, brain-derived EC (RBE4, GP8.39 and BBCEC) migration was inhibited by S1P (between 50 to 55%). In light of these results, and because it has already been shown that S1P promotes non cerebral angiogenesis, we can conclude that S1P is a specific inhibitor of brain angiogenesis.

Role of the receptor S1P-1 in the antiangiogenic effect of S1P in brain EC.

Because S1P-1 and S1P-3 are the two main receptors that mediate S1P angiogenic activity in EC, we wished to assess their contribution to the S1P-mediated inhibition of RBE4 cell migration and tubulogenesis. We used antisense oligonucleotides to downregulate S1P-1 and S1P-3 gene expression in RBE4 cells. Down-regulation of S1P-1 and S1P-3 gene expression was confirmed by RT-PCR (Fig.5A). Interestingly, S1P-1 downregulation antagonized the S1P-mediated inhibition of RBE4 cell migration (Fig.5B). However, no significant difference on

the S1P-inhibitory effect was observed between the control RBE4 cells and the S1P-3-lacking RBE4 cells. Consequently, we concluded that the inhibition of RBE4 cell migration by S1P is mainly mediated by the S1P-1 receptor. Semi-quantitative real-time PCR experiments were performed in order to determine the level of S1P-1 mRNA in RBE4 cells after incubation with antisense oligonucleotides. A mean decrease of 48% of the S1P-1 mRNA in RBE4 treated with the antisense oligonucleotides, was observed when compared to the untreated cells (data not shown). This correlates and confirms the downregulation of the S1P-1 mRNA observed by RT-PCR (Fig.5A). Furthermore, capillary-like tube formation of S1P-1-downregulated cells was also less effective (Fig.5C). Thus, we concluded that S1P-1 also regulates the antitubulogenic properties of S1P on brain EC.

Effect of the overexpression of S1P-1 on the response of brain EC to S1P.

Because S1P-1 appeared to be the main S1P receptor involved in the S1P-mediated inhibition of RBE4 cell migration, we overexpressed S1P-1 in RBE4 by transfection with a plasmid encoding for the fusion protein S1P-1-GFP and tested whether overexpression of S1P-1 could potentiate the S1P effect on RBE4 cell migration and tubulogenesis. Using fluorescence microscopy, we observed broad expression of the S1P-1-GFP fusion protein in RBE4 cells (Fig.6A). S1P-1-transfected RBE4 cells were allowed to migrate on fibronectin with varying concentrations of S1P. As expected, cells, which overexpressed S1P-1 underwent S1P-mediated migration inhibition at a lower S1P concentration than, did control cells (Fig.6B). Indeed, S1P-1-transfected RBE4 cell migration exhibited 50% inhibition at a S1P concentration of 0.027 μ M compared to 0.164 μ M for the control cells. Thus, S1P-1 overexpression sensitized RBE4 cells to the inhibitory effect of S1P. The same result was observed in tubulogenesis assays where the capillary-like structure formation of S1P-1-transfected RBE4 cells was more inhibited than in control cells (Fig.6C), as quantified in figure 6D. This clearly demonstrates that S1P-

1 triggers a specific transduction pathway leading to S1P-mediated brain antiangiogenic activity.

Expression of MT1-MMP in rat brain-derived EC.

MMPs have been shown to play a crucial role during angiogenesis. In particular, it has been shown that MT1-MMP, the best-characterized member of the MT-MMP family, is functionally involved in EC migration and tubulogenesis, as well as in the proMMP-2 activation process (Lafleur et al. 2003). MT1-MMP-dependent pro-MMP-2 activation in RBE4 cells was tested with ConcanavalinA (ConA) or CytochalasinD (CytoD) (Ailenberg and Silverman 1996; Eichmann et al. 2002), in parallel with U-87 glioblastoma cells. The extent of proMMP-2 activation in the conditioned media was assessed by gelatin zymography, as described in the Methods section. As shown in Figure 7A, ConA and CytoD treatments clearly induced proMMP-2 activation in U-87 cells, but failed to do so in RBE4 cells. Because the MT1-MMP-dependent proMMP-2 activation mechanism also involves TIMP-2, a tissue inhibitor of MMPs, we assessed the presence of this molecule in the conditioned media of various cell lines. As shown by Western blotting, TIMP-2 was found to be expressed in all cell lines tested, including RBE4 (Fig.7B). We then assessed transcription from the MT1-MMP gene by RT-PCR in various cell lines. MT1-MMP mRNA expression was absent in all the brain EC lines tested (RBE4, GP8.39), while MT1-MMP was clearly expressed in human glioma cells, rat CNS-1 glioma cells and non-cerebral human EC lines (HUVEC, HMEC-1) (Fig.7C).

Effect of the expression of MT1-MMP on the response of brain EC to S1P.

We hypothesized that the absence of MT1-MMP in brain EC could explain the antiangiogenic activity of S1P since intracellular crosstalk has been reported by us between S1P signaling and MT1-MMP expression (Annabi et al. 2003). Later, we have also shown that MT1-MMP cooperates with S1P to induce EC migration and

tubulogenesis (Langlois et al. 2004). In order to test this possibility, we transiently overexpressed MT1-MMP in RBE4 cells. The transfection efficiency was measured by Western Blot detection of MT1-MMP in RBE4 cell lysates (Figure 8A). The overexpression of MT1-MMP in RBE4 did not change the basal migratory potential of these cells (Fig.8B and 8C), which suggests that RBE4 cell migration does not depend on the presence of MT1-MMP. However, when transfected RBE4 cells migrated in the presence of S1P, we observed that the overexpression of MT1-MMP reversed the S1P-mediated inhibition of RBE4 cell migration (Fig.8C). The same result was observed for capillary-like structure formation ability on Matrigel™ (Fig.8D and 8E). Consequently, we conclude that the lack of MT1-MMP in brain EC explains the antiangiogenic effect of S1P.

DISCUSSION

Numerous molecules have been identified as playing crucial roles in the control of brain angiogenesis (Zadeh and Guha 2003). These include proangiogenic factors such as VEGF, bFGF and PDGF, as well as antiangiogenic factors (Distler et al. 2003). All these factors have been characterized in terms of modulating crucial cellular processes implicated in angiogenesis such as cell growth, cell differentiation or cell migration (Liekens et al. 2001). Lysophospholipids have also been identified as key modulators of the angiogenic process (Hla 2001). They have been shown to regulate numerous cellular mechanisms leading to angiogenesis (Panetti 2002). For instance, all of the studies of S1P activity in EC have indicated a proangiogenic role for this molecule (Lee et al. 1999). However, we present molecular and cellular evidences establishing that S1P can display tissue-specific antiangiogenic activities. Indeed, we show that S1P inhibits *in vitro* brain-derived EC migration and tubulogenesis. The specificity of the brain EC response to S1P stimulation could be explained by their unique function in the setting of the blood-brain barrier. In light of our results, the specific response of brain EC towards S1P clearly confirms that EC display phenotypical and molecular variability depending on their tissue location. We have previously provided molecular evidence of phenotypic distinction between tumoral and normal brain vasculature as well as between EC isolated from intracerebral and subcutaneous brain tumor models (Regina et al. 2003). These results indicated that the EC phenotype depends on interactions with surrounding cells but also with the ECM microenvironment. Thus, in the brain, EC experience the influence of various growth factors, which leads to the setting of a specific cerebral phenotype.

One pathophysiological consequence of the brain vascular phenotype may be found in inflammation, during which platelets secrete high quantities of S1P (English et al. 2000; Yatomi et al. 2000). The first event of the inflammatory response is a

strong vasodilatation. In peripheral vessels, S1P triggers the activation of EC, the release of NO and the relaxation of pericytes, leading to vasodilation (Igarashi et al. 2003; Nofer et al. 2004). However, S1P triggers a specific vasoconstriction of brain vessels, and could thus block the inflammatory process in the brain (Tosaka et al. 2001; Coussin et al. 2002; Salomone et al. 2003). Thus, it appears that the cerebral microenvironment strongly influences the endothelial response to S1P. Interestingly, S1P has been quantified in numerous tissues of various species (Wang et al. 1999; Murata et al. 2000; Yatomi et al. 2000) and a recurrent characteristic is its high quantity in brain. The specific localization of S1P in brain suggests that it could have a role during neurogenesis and brain angiogenesis (McGiffert et al. 2002). Our study presents strong evidence suggesting that S1P affects cerebral EC angiogenic properties, and indicates that S1P could stabilize the brain vasculature through its antiangiogenic effect on brain EC. This raises an attractive hypothesis of a possible protective role for S1P in brain, as already described in lung (Peng et al. 1993; Schaphorst et al. 2003).

At the molecular level, we determined that the lack of MT1-MMP at the brain EC surface was responsible for the antiangiogenic activity of S1P. The role of MT1-MMP in the S1P-mediated angiogenic activity has already been documented by us (Annabi et al. 2003; Langlois et al. 2004). S1P has been shown to be proangiogenic for numerous cell lines. It has also been shown that S1P increases HUVEC, HMEC-1, BAEC and BMSC (*bone marrow-derived stem cell*) cell migration and tubulogenesis (Lee et al. 1999; Annabi et al. 2003). One common feature in S1P-responsive EC is that they all express MT1-MMP. The brain EC, which do not express MT1-MMP, are characterized by their antiangiogenic response to S1P. It is thus tempting to suggest that the expression of MT1-MMP in EC modulates the angiogenic potential of EC in response to S1P.

S1P has already been shown to affect capillary-like tube formation in various non-cerebral EC (Lee et al. 1999; Annabi et al. 2003; Bayless and Davis 2003). We have shown that BAEC, which express low levels of MT1-MMP, display a strong migration in response to S1P. Using the same cells, we have demonstrated that BAEC do not undergo morphogenic differentiation in response to S1P (Langlois et al. 2004). Thus, it seems that low expression of MT1-MMP is sufficient to trigger a pro-migratory response to S1P but is not sufficient to induce endothelial tubulogenesis in response to S1P. Accordingly, overexpression of MT1-MMP in BAEC triggered the EC morphogenic differentiation in response to S1P (Langlois et al. 2004). Then, we can safely state that MT1-MMP expression dictates the angiogenic response to S1P in EC.

The tissue-specific expression of MT1-MMP described here leads to reconsideration of the role of MT1-MMP in endothelial morphogenic differentiation. Numerous papers have shown that MT1-MMP plays a major role in endothelial tubulogenesis (Chiarugi et al. 2000; Hotary et al. 2000; Lamy et al. 2006). In the present study, we clearly observe that brain EC display potent MT1-MMP-independent tubulogenesis. We cannot, however, preclude that other MT-MMP isoforms may compensate for the absence of MT1-MMP (Lafleur et al. 2002). It is well known that a variety of MT-MMPs exist, with different tissue distributions. Our study supports previous results concerning the specialized location of certain MT-MMPs. The absence of MT1-MMP could be one of the molecular characteristics of the brain EC phenotype that may regulate some of the blood brain barrier functions. Other MT-MMPs have been shown to be expressed in a tissue-dependent manner. For instance, MT5-MMP have been described as being mainly expressed in brain (Hayashita-Kinoh et al. 2001; Sekine-Aizawa et al. 2001). Thus, assessing the roles of other MT-MMPs, and more particularly MT5-MMP, in cerebral EC should certainly provide new insights in our understanding of the major role that these proteins play in brain angiogenesis.

We have shown that the expression of the MT1-MMP in brain EC antagonizes the specific S1P antiangiogenic activity. The differential effect observed between control and MT1-MMP expressing brain EC clearly demonstrates the ability of MT1-MMP to transduce intracellular signaling pathways regulating angiogenic processes. However, the specific MT1-MMP-mediated transduction pathway, leading to the proangiogenic response to S1P, remains to be elucidated. Altogether, the present data revealed the antiangiogenic role of S1P in brain. According to the major role of cerebral endothelium in the setting of the blood brain barrier, we suggest that S1P, in physiological conditions, acts as a stabilizing agent of the blood-brain-barrier by inhibiting brain EC migration. Because of its dose-dependent activity, S1P should be more active on brain EC in case of inflammation than in physiological conditions. Consequently, S1P might also participate to a global brain protection process during pathological conditions through a stronger stabilization of the blood brain barrier.

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FIGURE LEGENDS

Figure 4.1: Fibronectin is the most potent ECM substrate for RBE4 cell adhesion and migration (A) Cells (5×10^4) were seeded on 96-well plates previously coated with various purified ECM proteins, and allowed to adhere for 2 hours at 37°C. (B) Chemotaxis assays were performed as described in the Material and Methods section. The lower faces of the Transwells were previously coated with either type I collagen (CO), fibronectin (FN), vitronectin (VN) or laminin-1 (LN). Cells that migrated to the underside were fixed, stained and counted. Example data of a significant assay is presented. (C) Bar graph represents a mean quantification of at least three independent experiments.

Figure 4.2: RBE4 cell migration on fibronectin is inhibited by S1P The lower faces of the Transwells were previously coated with 10 µg/ml fibronectin. Cells (10^5) were seeded on the upper face of Transwells and allowed to migrate for 1 hour in a serum-free medium containing one of the following growth factors: S1P (10 µM), PDGF (10 ng/ml), VEGF (10 ng/ml), bFGF (10 ng/ml), TNFα (50 ng/ml) and GM-CSF (50 ng/ml). As a positive control, cells were allowed to migrate towards 10% FBS-containing medium. Cells that migrated to the underside were counted. ★ indicates statistically significant differences ($P < 0.05$) compared with the control condition (serum-free medium) using Student's *t*-test.

Figure 4.3: S1P inhibits RBE4 cell migration and *in vitro* tubulogenesis. (A) Migration assays were performed as described in the Materials and Methods section. Increasing concentrations of S1P were added to the lower compartment of the Transwells. (B) A representative tubulogenesis assay is shown after treatment of RBE4 with varying concentrations of S1P. Morphogenic differentiation assays were performed as described in the Materials and Methods section. Cells (5×10^4) were added to the Matrigel-coated wells in serum-free medium containing increasing S1P

concentrations. (C) The capillary-like structures formed in the gel were quantified and a mean quantification is presented. ★ indicates statistically significant differences ($P<0.05$) compared with the control assay (serum-free medium) using Student's *t*-test.

Figure 4.4: S1P specifically inhibits the migration of brain derived-EC Non-cerebral EC (BAEC, HMEC-1 and HUVEC) and cerebral EC (RBE4, GP8.39 and BBCEC) were harvested by brief trypsinization. Migration assays were performed on fibronectin coated-Transwells. Cells (10^5) of each cell line were added to the wells and allowed to migrate in a serum-free medium containing or not 10 μ M S1P. Results are expressed as the fold-induction of cell migration over basal migration of untreated cells.

Figure 4.5: S1P-1 transduces the antiangiogenic effects of S1P in brain endothelial cells RBE4 cells were transiently treated with 200 nM of antisense (AS) oligonucleotides against S1P-1, S1P-3, or with their scrambled controls (S), and used 72 hours post-transfection. (A) Gene expression level of S1P-1 and S1P-3 were assessed in transfected RBE4 cells by RT-PCR. β -actin cDNA was amplified as a loading control. (B) Cell migration assays were performed as described in the Materials and Methods section. Transfected RBE4 cells were allowed to migrate on fibronectin-coated Transwells in serum-free medium containing (or not) 10 μ M S1P. (C) Control and transfected cells were added to Matrigel™ coated-wells for 18h at 37°C with or without 10 μ M S1P. After this time, capillary-like structures were quantified. ★ represents statistically significant differences ($P<0.05$) compared to the untreated control cells. ★★ indicate statistically significant differences ($P<0.05$) compared to the S1P-treated control cells.

Figure 4.6: Overexpression of S1P-1 in RBE4 cells potentiates the S1P antiangiogenic effect RBE4 cells were transiently transfected with a cDNA plasmid encoding S1P-1-GFP or the pcDNA (3.1+) empty plasmid (mock). (A) Phase contrast (PC) and fluorescence views of mock or S1P-1-GFP transfected RBE4 cells. (B) Effect of overexpression of S1P-1 on S1P-mediated RBE4 cell migration. (C) S1P-1-transfected RBE4 cell tubulogenesis was tested on Matrigel™. Cells were plated on Matrigel for 18h, with or without 10 μ M S1P. Example data are presented. (D) Tube formation was measured and a mean quantification of at least three independent experiments is presented. ★ indicates statistically significant differences ($P < 0.05$) compared with the control assay (serum-free medium) using Student's *t*-test.

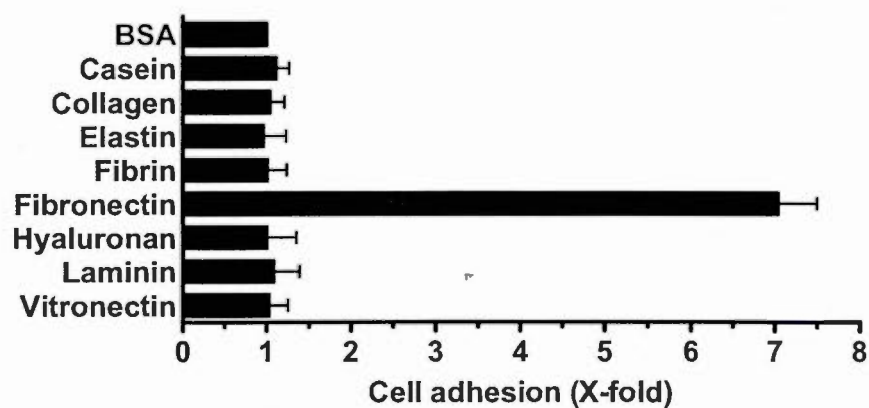
Figure 4.7: Pro-MMP-2 activation, TIMP-2 and MT1-MMP expression in rat brain endothelial cells (A) U-87 glioblastoma cells and RBE4 were treated with either ConA (10 μ g/ml) or CytoD (1 μ M) for 18h. ProMMP-2 activation in the conditioned media was then assessed by gelatin zymography. (B) TIMP-2 expression in U-87, CNS-1, RBE4 and GP8.39 conditioned media was detected by Western Blot analysis. (C) RT-PCR experiments were performed in order to verify the expression of MT1-MMP in various cell lines. Total RNA was extracted as described in the Materials and Methods section and a MT1-MMP cDNA fragment was amplified using specific oligonucleotides. BEC represents isolated EC from normal rat brain. β -actin cDNA was amplified as a loading control.

Figure 4.8: Overexpression of MT1-MMP antagonizes S1P-mediated antiangiogenic effects on RBE4 cells RBE4 cells were transfected with MT1-MMP cDNA or the corresponding empty vector (mock). (A) MT1-MMP expression in RBE4 cell lysates was measured by Western Blot analysis. N.S. Non specific immunoreactive band. (B) Representative datas of MT1-MMP overexpressing RBE4 cells. 10^5 cells were allowed to migrate on fibronectin-coated Transwells, with 10 μ M

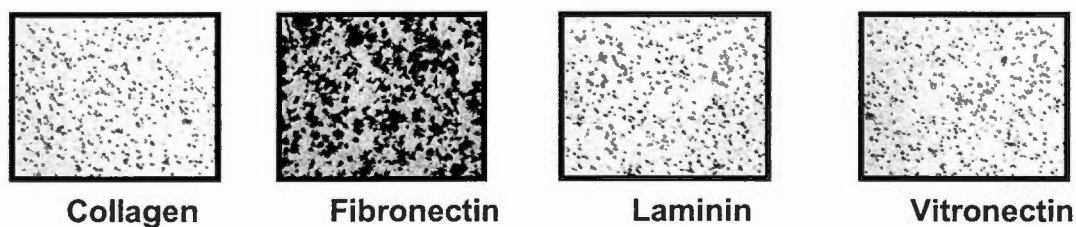
S1P, for 1h at 37°C. (C) Mean quantification of the migratory potentials of transfected RBE4 cells. (D) Example data of tubulogenesis assays. Control and transfected cells were added to Matrigel™ coated-wells for 18h at 37°C, in the presence or absence of 10μM S1P. (E) Mean quantification of capillary-like structures. ★ represents statistically significant differences ($P<0.05$) compared to the untreated control cells.

Figure 1: Fibronectin is the most potent ECM substrate for RBE4 cell adhesion and migration

A)



B)



C)

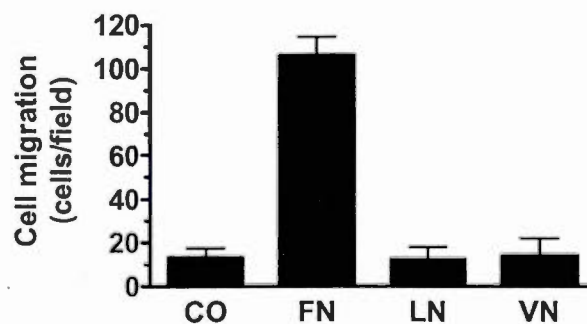


Figure 2: RBE4 cell migration on fibronectin is inhibited by S1P

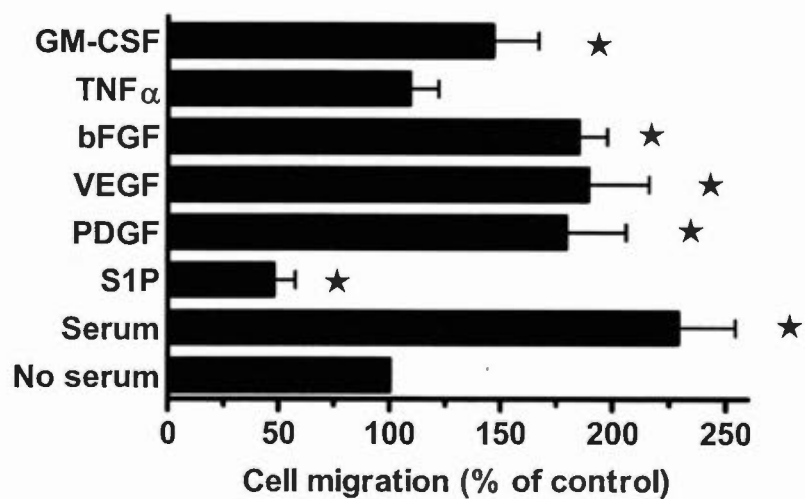
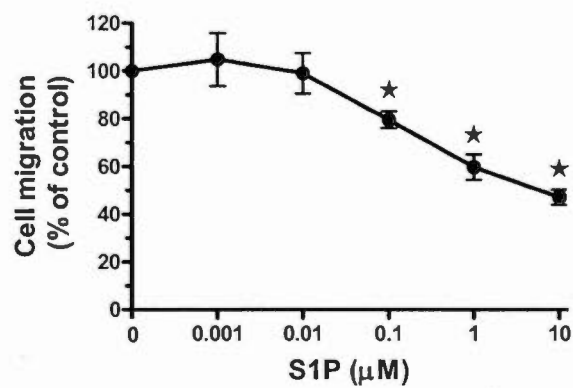
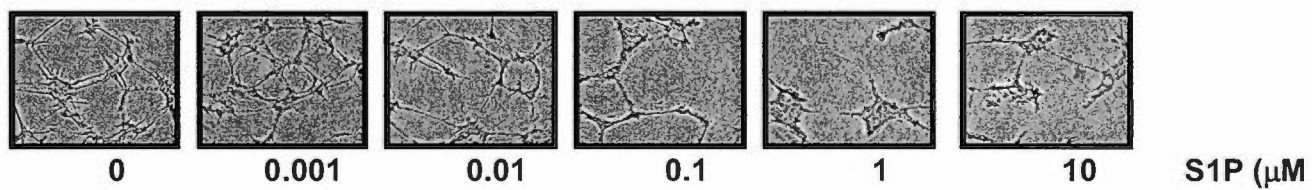


Figure 3: S1P inhibits RBE4 cell migration and *in vitro* tubulogenesis

A)



B)



C)

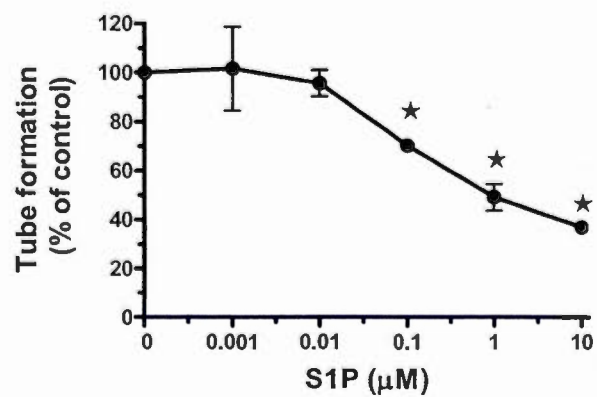


Figure 4: S1P specifically inhibits the migration of brain derived-EC

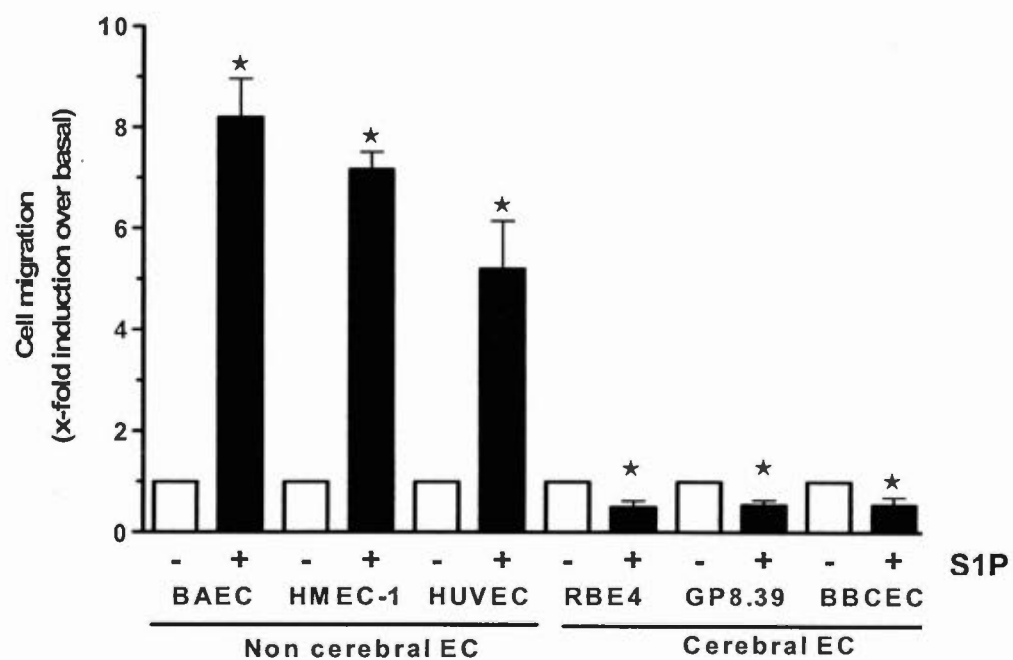
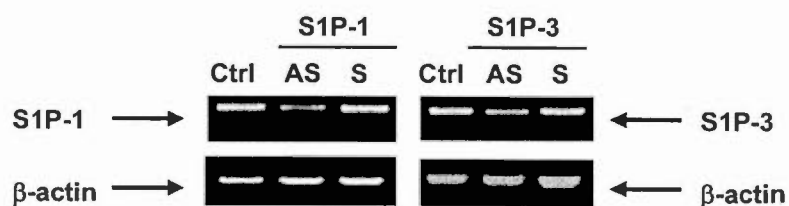
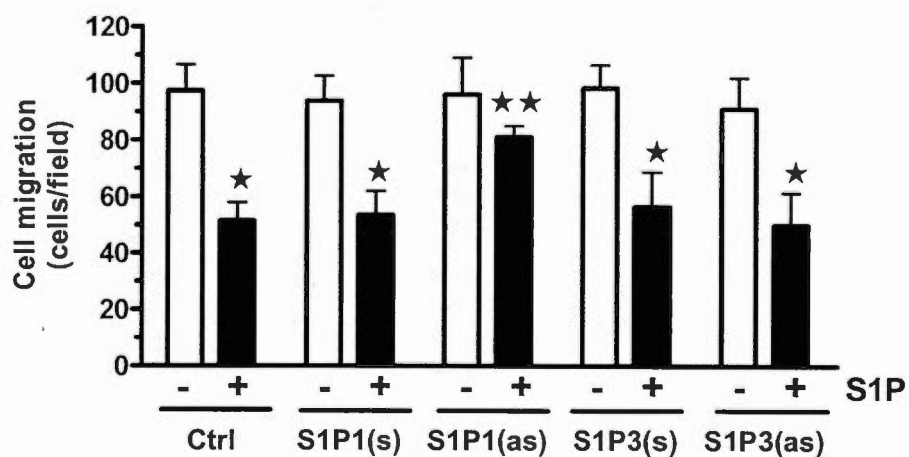


Figure 5: S1P-1 transduces the antiangiogenic effects of S1P in brain endothelial cells

A)



B)



C)

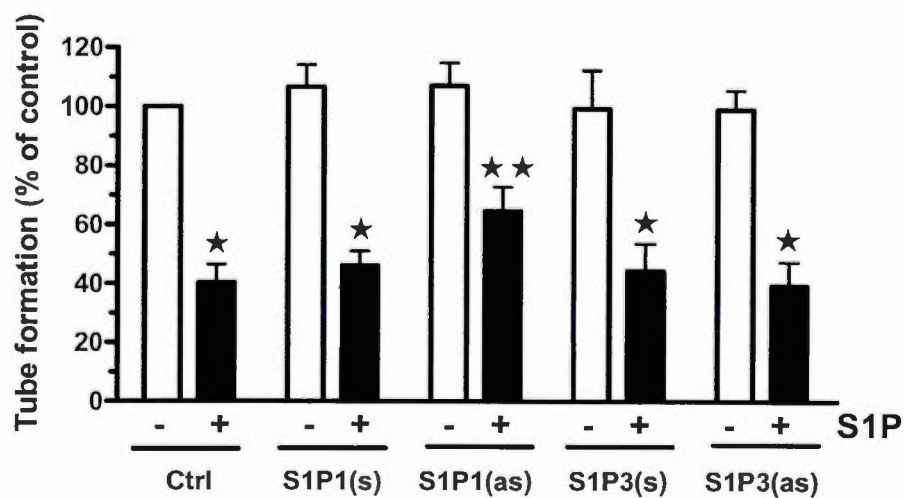
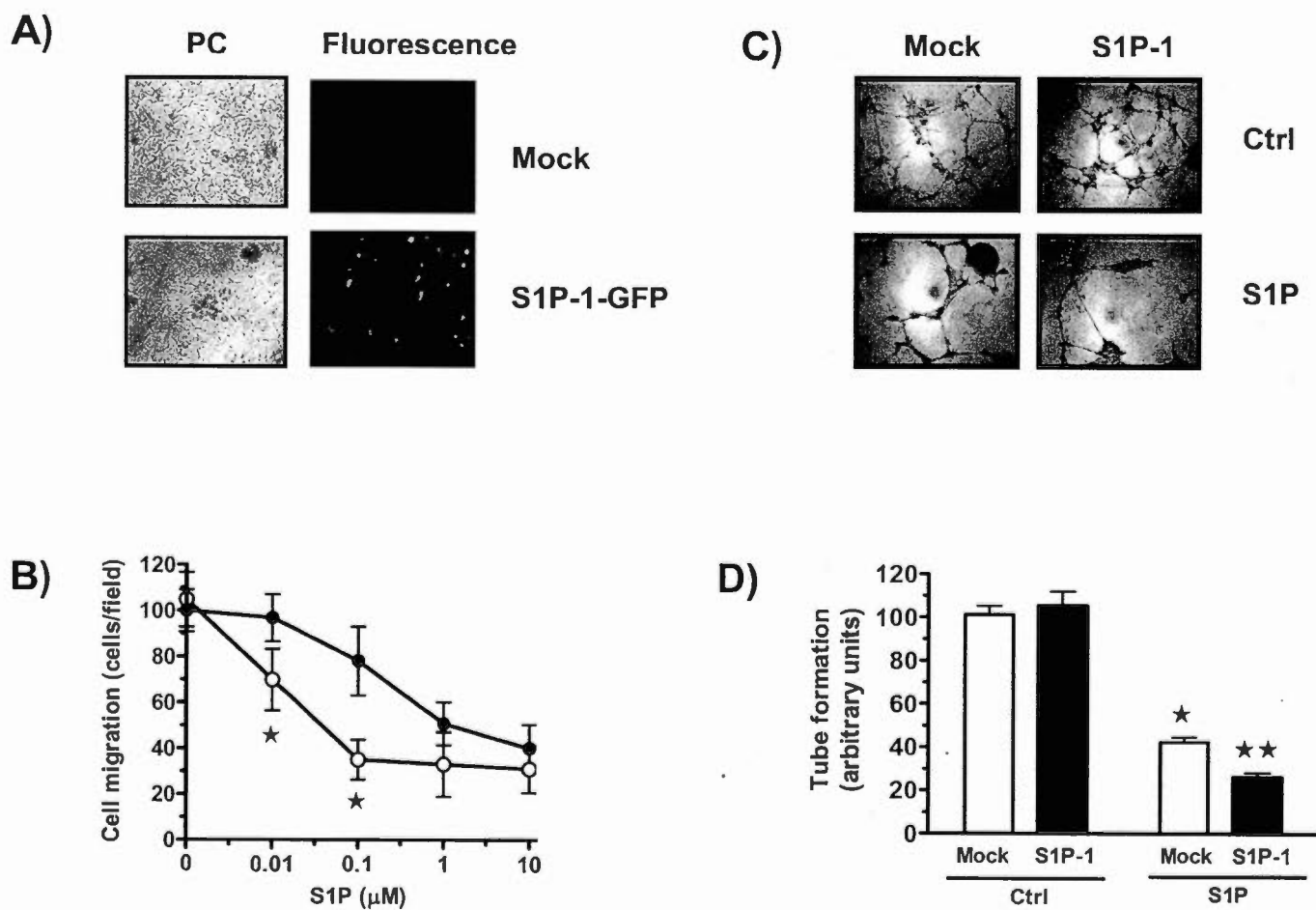
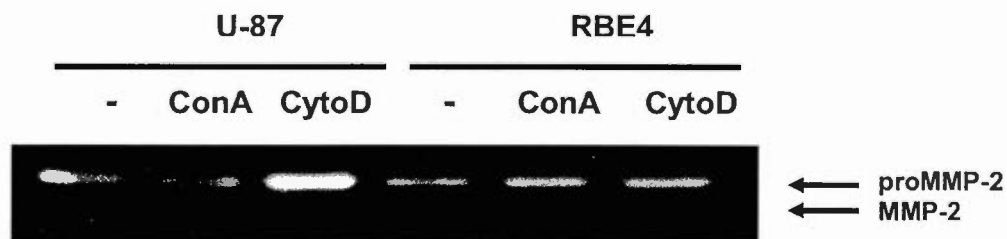


Figure 6: Overexpression of S1P-1 in RBE4 cells potentiates the S1P antiangiogenic effect

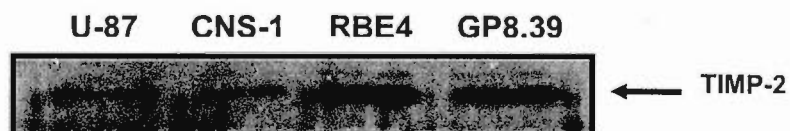


**Figure 7: Pro-MMP-2 activation, TIMP-2 and MT1-MMP
Expression in rat brain endothelial cells**

A)



B)



C)

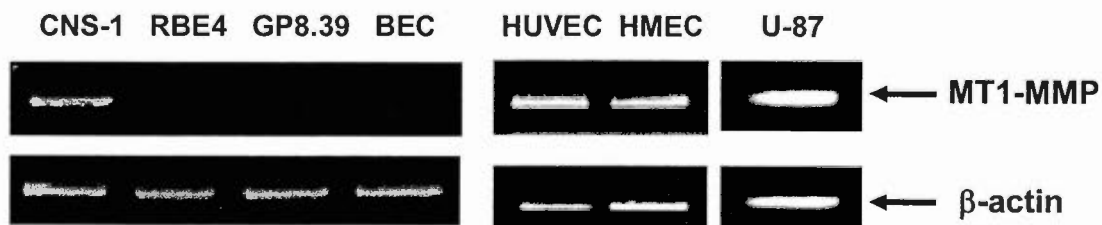
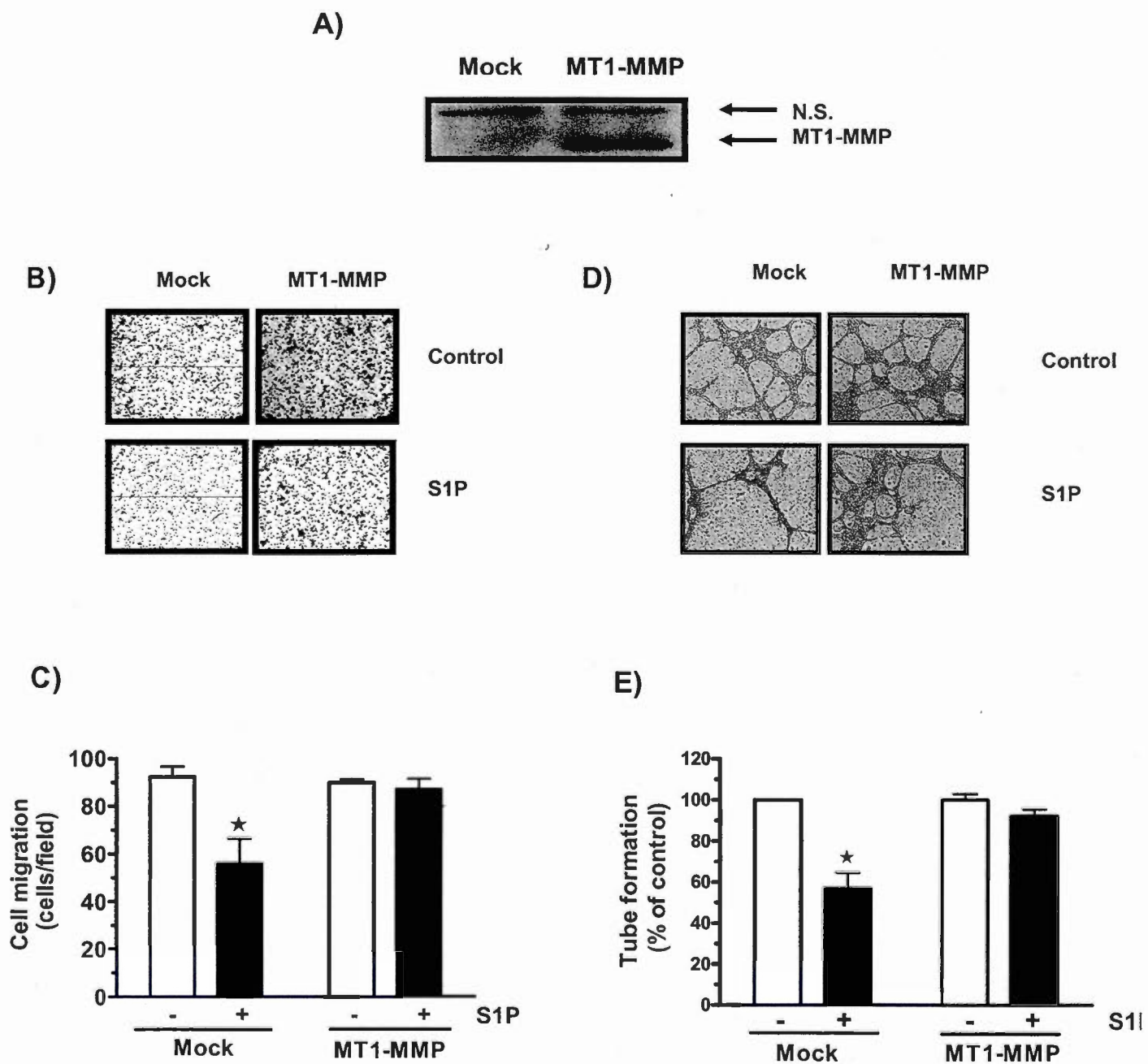


Figure 8: Overexpression of MT1-MMP antagonizes S1P-mediated antiangiogenic effects on RBE4 cells



Article 4 : Modulation of P-gp function by sphingosine kinase-1 in brain endothelial cells

Anthony Pilorget^{1,2}, Michel Demeule¹, Stéphane Barakat¹, Jacques Marvaldi², José Luis² and Richard Béliveau^{1*}.

¹*Laboratoire de Médecine Moléculaire, Centre de Cancérologie Charles-Bruneau, Hôpital Sainte-Justine-UQAM, Montreal, Quebec, Canada and* ²*CNRS FRE 2737, Faculté de Pharmacie, 27, bd Jean Moulin, 13385 Marseille, Cedex 5, France.*

* To whom correspondence and reprint requests should be directed

Laboratoire de Médecine Moléculaire

Université du Québec à Montréal

C.P. 8888, Succursale Centre-ville

Montréal, Québec

CANADA H3C 3P8

Phone : (514) 987-3000 ext 8551

Fax : (514) 987-0246

E-mail : oncomol@nobel.si.uqam.ca

Running title: Sphingosine kinase-1 stimulates P-gp transport activity.

Abbreviations used: SphK: sphingosine kinase, S1P: sphingosine-1-phosphate, P-gp: P-glycoprotein, MDR: multidrug resistance, EC: endothelial cells, Vb: vinblastine, GFP: green fluorescent protein, PTX: pertussis toxin.

CONTRIBUTION

Ce quatrième article a mis en évidence l'importance de la sphingosine kinase (SphK) et de son produit la sphingosine-1-phosphate (S1P) dans la régulation de l'expression et de l'activité de transport de la glycoprotéine P (P-gp) dans les cellules endothéliales cérébrales de rat RBE4. Nos résultats démontrent notamment que la SphK et la S1P stimulent fortement l'activité de transport de la P-gp. De plus, nous démontrons que la SphK stimule aussi l'expression de la P-gp. Enfin, il apparaît que les récepteurs S1P-1 et S1P-3 jouent un rôle majeur dans la régulation de ces phénomènes. Cet article est le premier à mettre en évidence le rôle crucial du couple SphK/S1P dans le contrôle de la P-gp.

J'ai réalisé l'ensemble des expériences, en collaboration avec Stéphane Barakat, étudiant en thèse au sein de l'unité CNRS UMR 5086 à Lyon (France). Ces travaux ont été réalisés sous la supervision directe du Dr Michel Demeule et de mes deux co-directeurs, le Dr Richard Béliveau et le Pr José Luis. J'ai rédigé cet article, qui a été relu par par le Dr Anthony Régina, le Pr José Luis et le Dr Richard Béliveau. Cet article a été accepté pour publication dans la revue scientifique *Journal of Neurochemistry* en août 2006.

RÉSUMÉ EN FRANÇAIS

La glycoprotéine P (P-gp) est un transporteur de la famille ABC exprimée dans les capillaires cérébraux, et dont le rôle est d'extruder les xénobiotiques afin de protéger le cerveau. Cependant, sa surexpression a été associée avec le phénotype de résistance multiple aux médicaments (MDR) dans les tumeurs. Dans cet article, nous avons étudié la régulation de l'activité de transport de la P-gp par la sphingosine kinase-1 (SphK-1) dans des cellules endothéliales (CE) cérébrales de rat RBE4. Nous démontrons d'abord que l'expression de la SphK-1 est plus élevée dans les CE isolées de tumeurs cérébrales que dans les CE isolées de cerveaux sains. Nous démontrons aussi que la surexpression de la SphK-1 dans les cellules RBE4 entraîne la surexpression de la P-gp tant au niveau génique que protéique, et que cette surexpression est dépendante de l'activité catalytique de la SphK-1. De plus, nous avons déterminé les effets de la S1P, le produit de la SphK-1, sur les fonctions de la P-gp. La S1P stimule fortement l'activité de transport de la P-gp, sans pour autant moduler son expression. Finalement, nous avons découvert que la S1P agissait sur la P-gp via les récepteurs S1P-1 et S1P-3 présents à la surface des cellules RBE4. L'ensemble de ces résultats indique que la SphK-1 et son produit la S1P sont impliqués dans le contrôle de l'activité de la P-gp dans les cellules RBE4. Puisque la SphK-1 est surexprimée dans les CE issues des tumeurs cérébrales, ces résultats suggèrent que cette kinase et son produit pourrait contribuer à la mise en place et au maintien du phénotype MDR dans les CE cérébrales.

ABSTRACT

P-glycoprotein (P-gp), an ABC-transporter highly expressed in brain capillaries, protects the brain by extruding xenobiotics. However, its overexpression has also been associated with the multidrug resistance (MDR) phenotype in tumors. Here, we have investigated the regulation of P-gp transport activity by sphingosine kinase 1 (SphK-1) in rat brain endothelial cells. We first demonstrate that SphK-1 is overexpressed in endothelial cells (EC) isolated from rat brain tumors compared to EC from normal brain. We also provide evidence that the overexpression of SphK-1 in the cerebral EC line RBE4 leads to the upregulation of P-gp, both at the gene and protein levels, and that this modulation depends on the catalytic activity of SphK-1. Moreover, we determined the effect of S1P, the product of SphK-1, on P-gp function. S1P strongly stimulates P-gp transport activity, without modulating its expression. Finally, we found that the S1P-mediated stimulation of P-gp activity is mediated by S1P-1 and S1P-3 receptors at the RBE4 cell surface. Altogether, these results indicate that SphK-1 and its product S1P are involved in the control of P-gp activity in RBE4 cells. Since SphK-1 is overexpressed in EC from brain tumors, these data also suggest that this kinase and its product could contribute to the acquisition and the maintenance of the multidrug resistance phenotype in brain tumor-derived endothelial cells.

INTRODUCTION

P-glycoprotein (P-gp) was first studied in the context of cancer research where its overexpression in tumor cells has been associated with the multidrug resistance (MDR) phenotype (Henson et al. 1992). The multidrug resistance P-gp is encoded by the *MDR1* gene in humans, whereas two genes encode paralogous proteins in rodents, *mdr-1a* and *mdr-1b* (Ueda et al. 1986; Devault and Gros 1990). In cancer cells, P-gp acts as an efflux pump that extrudes chemotherapeutic agents out of the cells, decreasing their intracellular concentration (See et al. 1974; Ozben 2006). High levels of P-gp expression have been observed in the endothelial cells (EC) of brain capillaries (Regina et al. 2001; Okaji et al. 2006), mainly at the luminal side of the brain vascular endothelium (Beaulieu et al. 1997). P-gp expressed in brain EC aims to protect the brain against xenobiotics that could affect normal brain functions. Several proteins, such as PKC, caveolin-1 or HIV-Tat, have been shown to regulate P-gp function (Demeule et al. 2002b; Hayashi et al. 2005; Tabe et al. 2006). However, few studies have investigated the role of the sphingosine kinase and its product sphingosine-1-phosphate (S1P) in the control of the P-gp function.

The lysophospholipid S1P is released in case of inflammation by activated platelets (Billich et al. 2005) and was found to act as an *extracellular* mediator by binding to specific members of the EDG-family of G-protein coupled receptors (GPCRs, originally named EDG-1, -3, -5, -6, and -8 and now termed S1P-1 to -5 receptors). In addition to its function as a ligand for cell surface receptors, S1P also acts as an intracellular messenger (Pettus et al. 2002). S1P is produced by the phosphorylation of sphingosine, catalyzed by sphingosine kinase (SphK), the most distal enzyme in the sphingomyelin pathway of sphingolipid degradation. To date, two distinct SphK isoforms, SphK-1 and SphK-2, have been cloned and characterized (Maceyka et al. 2005a). SphK-1 has been shown to be upregulated in various tumors (French et al. 2003). The strong expression of SphK-1 is also associated to the

sustained growth of tumor cells through the S1P-mediated inhibition of tumor cell apoptosis (Maceyka et al. 2002). Recently, the expression of SphK-1 has been associated to a poor survival prognosis for patients with brain tumor (Van Brocklyn et al. 2005). Moreover, it has been shown that SphK-1 impairs the efficacy of chemotherapy in prostate adenocarcinoma cells (Pchejetski et al. 2005). Since P-gp is also associated to a poor prognosis for patients with a brain tumor and plays a major role in the chemotherapy efficacy (Regina et al. 2001), we hypothesize that SphK-1 and its product S1P, could regulate P-gp transport activity.

In the present paper, we demonstrate that SphK-1 is upregulated in brain endothelial cells isolated from brain tumors compared to normal brain EC. We also provide evidence that SphK-1 and its product S1P modulate P-gp transport activity in the rat brain endothelial cell line RBE4. Moreover, our data support the hypothesis that P-gp transport activity is regulated by S1P. Finally, we demonstrate that extracellular S1P mediates its effects on P-gp function by interacting with S1P-1 and S1P-3 on the RBE4 cell surface. Altogether, our results suggest that the SphK-1/S1P couple participates to the regulation of P-gp function in endothelial cells of the blood brain barrier.

MATERIAL AND METHODS

Antibodies and chemicals: TriZOL reagent, trypsin and geneticin (G418 were from Invitrogen (Burlington, ON). S1P, indomethacin, probenecid, verapamil, gentamycin, sodium dodecyl sulfate (SDS), trypsin for EC and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). N,N dimethylsphingosne was from Cedarlane laboratories (Hornby, ON). Cyclosporin A (CsA) and SDZ PSC833 were provided by Novartis Pharma Canada Inc. (Dorval, QC, Canada). bFGF was from Upstate Biotechnology (Lake Placid, NY). Epithelial growth factor (EGF) was from BD Biosciences Labware (Bedford, MA). Type-1 collagen was extracted from rat tail tendon by standard method (Silver and Trelstad 1980). mAb against P-gp (namely mAb C219) was from ID Labs (London, ON, Canada). The rabbit polyclonal anti-SphK-1a antibody was a kind gift of Dr Yoshiko Banno. Secondary antibodies, linked to horseradish peroxidase, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and Enhanced Chemiluminescence Reagent Plus (ECL) reagents were from NEN™ Life Science Products (Boston, MA).

Cells and culture media: α -MEM medium, Ham's F12 Nutrient Mixture and L-Glutamine were purchased from GIBCO BRL (Burlington, ON). Fetal calf serum (FBS) was from HyClone Laboratories (Logan, UT). RBE4 immortalized rat brain microvessel EC were gift of Dr Françoise Roux. RBE4 cells were plated on type I collagen-coated plates (200 μ g/ml) and maintained in α MEM/Ham's F12 (1:1 v/v) containing 10% heat-inactivated FCS, 30 mg/ml geneticin, 50 mg/ml gentamycin and 100 ng/ml bFGF. All cells were incubated at 37°C, air/CO₂ (95%/5%).

Isolation of EC fractions from brain tissue: The isolation of EC from normal male Lewis rat (Charles River, QC, Canada) brains has been performed as previously described (Regina et al. 2003). All animal experiments were evaluated and approved

by the Institutional Comity for Good Animal Practices (UQAM, Montréal, QC, Canada). Magnetic cell sorting (MACS) was performed using microbeads, a MidiMacs separation unit and positive selection MACS columns from Miltenyi Biotec (Auburn, CA, USA). Mouse anti-PECAM-1 antibody that was linked to microbeads was from Cedarlane Laboratories (Hornby, ON, Canada).

cDNA constructs and cell transfection: The plasmid encoding for the mouse sphingosine kinase-1-GFP chimera was generously provided by Dr Sarah Spiegel. The dominant negative form of the human sphingosine kinase-1-GFP (G82D) chimera was a kind gift of Dr Lina Obeid. RBE4 cells were transiently transfected with cDNA plasmids by using the liposomal formulation TranspassD1 transfection reagent (New England Biolabs inc., Pickering, ON). Transfection efficiency was confirmed by western blotting. All experiments involving transfected cells were performed 36 hours following transfection. Mock transfection of RBE4 cells with pcDNA (3.1+) expression vector alone was used as control.

Sphingosine-1-phosphate measurement: ^{32}P i was purchased from Amersham (Baie d'Urfé, QC). RBE4 cells were washed with phosphate-free medium and incubated with 2 ml of this buffer containing ^{32}P i (40 $\mu\text{Ci/ml}$) for 16 h in a 6-well plate. Placing the dishes on ice, and rapidly removing the medium terminated the reactions. The cells were scraped from the dish in 1 ml of 0.1 N HCl. Samples were then supplemented with 2.5 volumes of chloroform/1-butanol/HCl (50/50/1 v/v), vortexed for 2 min, and incubated at room temperature for 10 min. Samples were spun at $5,000 \times g$ for 10 min. Organic phases were dried under nitrogen and analyzed by TLC. This single step extraction allowed the extraction of S1P with an efficiency of >90% recovery (Ancellin et al. 2002). Samples were resuspended in 50 μl of chloroform. [^{32}P]S1P was resolved by TLC on Silica Gel G60 using 1-butanol/acetic acid/water (60/20/20 v/v) buffer. Bands corresponding to S1P, revealed by the

ninhydrine staining of purified S1P, were scraped from the plates and counted in a scintillation counter.

Immunoblotting procedures: Cells were homogenized in lysis buffer. 30 μ g of protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE). All samples, except for P-gp detection, were boiled for 5 minutes at 105°C. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween 20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies in TBST containing 3% BSA, followed by a 1 hour incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/5,000 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by ECL+.

Total RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was extracted from control and transfected RBE4 cells using the TRIzol Reagent. RT-PCR reactions were performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq Kit from Invitrogen (Burlington, ON). RT-PCR reactions were performed with specific oligonucleotide primers derived from rat sequences and PCR conditions were optimized so that the gene products were obtained during the exponential phase of the amplification. PCR products were resolved on 2% (w/v) agarose gels containing 1 μ g/ml ethidium bromide.

Vinblastine accumulation assay. Cells were washed twice with HBSS (1.3 mM CaCl_2 , 5.4 mM KCl, 0.44 mM KH_2PO_4 , 0.5 mM MgCl_2 , 0.83 mM MgSO_4 , 137 mM NaCl, 4.2 mM NaHCO_3 , 0.34 mM Na_2HPO_4 , and 25 mM D-glucose, pH 7.3) then incubated for 1 h at 37°C in HBSS containing 50 nM [^3H]-Vb from Amersham (Baie d'Urfé, QC). [^3H]-Vb accumulation was stopped by washing the cells 5 times

with ice-cold HBSS. Cells were lysed with 0.1% Triton X-100 at room temperature overnight or with 0.1 N NaOH for 1 hour. 200 µl of each sample were mixed with 10 ml scintillation liquid and the radioactivity in the cell lysate was measured. Protein concentration was determined in each samples and radioactivity was normalized on the protein concentration.

Treatment with antisense oligonucleotides: Antisense phosphorothioate oligodeoxyribonucleotides (PTO) against S1P-1, S1P-3 and their respective scrambled controls were custom synthesized based on published sequences (Lee et al. 1999; Liu et al. 2001). The sequences of the oligonucleotides used are the following: S1P-1 antisense, 5'-GAC GCT GGT GGG CCC CAT-3'; scrambled S1P-1, 5'-TGA TCC TTG GCG GGG CCG-3'; S1P-3 antisense, 5'-CGG GAG GGC AGT TGC CAT-3'; scrambled S1P-3, 5'-ATC CGT CAA GCG GGG GTG-3'. To reduce S1P-1 and S1P-3 gene expression, RBE4 cells were grown to approximately 60% of confluence and incubated with 200 nM of either the scrambled controls or the S1P-1 or S1P-3 antisense oligonucleotides using Transpass D1 transfection reagent. Cells were used 72h after transfection.

RESULTS

Upregulation of SphK-1 in EC isolated from brain tumor. SphK-1 expression has been shown to be correlated with a poor survival of patients with glioblastoma multiforme (Van Brocklyn et al. 2005). Then, we were interested to measure the expression of SphK-1 in EC derived from normal brain and glioma. By using a well-established protocol (Regina et al. 2003), EC from normal rat brain and glioma were isolated. The expression of SphK-1 in these lysates was evaluated by Western blot analysis. As shown on figure 1A, the expression of SphK-1 in glioma-derived EC is much higher than in normal brain-derived EC. By densitometric analysis, we measured a three-fold increase of SphK-1 expression in glioma-derived EC compare to normal brain (Figure 1B).

Overexpression of SphK-1-GFP in RBE4 cells: RBE4 cell line was used as a model of brain EC. These cells were transfected with either a mock plasmid or a plasmid coding for the wild-type GFP-tagged form of the SphK-1. The expression of the SphK-1-GFP protein in RBE4 cells was evaluated by fluorescence microscopy. As shown on figure 2A, the SphK-1-GFP plasmid transfection is merely effective in RBE4 cells. Afterwards, the concentration of intracellular and extracellular S1P was measured, as described in the methods section (Fig.2B). We can note that the vast majority of S1P remains within the cell, whereas a small percentage is excreted in the cell environment (Fig.2B). When SphK-1-GFP was overexpressed in RBE4 cells, both the intracellular and the extracellular form of S1P was increased (Fig.2B). These results indicate that this model, in spite of the fact that the transfection efficiency is around 20%, can be used to assess the effect of SphK-1 on P-gp expression and activity.

Stimulation of P-gp expression in SphK-1-transfected RBE4 cells: Because both SphK-1 and P-gp expression are associated to a poor survival prognosis in patients

with brain tumor, we investigated the effect of the overexpression of SphK-1 on P-gp expression in RBE4 cells. By Western blot, the expression of SphK-1 in control and SphK-1-GFP transfected RBE4 cells was determined. In control and SphK-1-GFP transfected cells, a band at around 40 kDa, corresponding to the basal expression of SphK-1 in RBE4 cells (Fig.3A) was detected. In SphK-1-GFP transfected cells, a second band, at around 70kDa, corresponding to the GFP-tagged SphK-1 protein was also observed (Fig. 3A). When P-gp was detected in the same lysates, we determined a 2-fold increase in the expression of the protein (Fig. 3A).

In order to better characterize this phenomenon, we performed RT-PCR analysis of the expression of both *mdr-1a* and *mdr-1b* genes in mock and transfected cells. As shown in figure 3B, the overexpression of the wild-type form of the SphK-1 protein led to the upregulation of the *mdr-1b* gene, without affecting the expression of *mdr-1a* gene. However, the expression of both *mdr-1a* and *mdr-1b* was unaffected by the transfection of the dominant negative form of SphK-1 (G82D) (Fig. 3B). This indicates that the regulation of the P-gp expression by SphK-1 depends on the catalytic activity of SphK-1.

Stimulation of P-gp transport activity in SphK-1 transfected RBE4 cells: Since the overexpression of SphK-1 in RBE4 cells induced P-gp expression, we further investigated the impact of this transfection on P-gp transport activity. We tested the accumulation of vinblastine ($[^3\text{H}]\text{-Vb}$) in RBE4. As shown on figure 4A, the blocking of $[^3\text{H}]\text{-Vb}$ efflux by P-gp inhibitors (verapamil, cyclosporine A and PSC833) led to an increase in its intracellular accumulation. However, broad-spectrum inhibitors of MRPs (probenecid and indomethacin) did not affect the accumulation of $[^3\text{H}]\text{-Vb}$ in RBE4, suggesting that vinblastine, under the present experimental conditions, is specifically extruded by P-gp in RBE4 cells. Afterwards, we investigated the effect of SphK-1 overexpression on the P-gp dependent $[^3\text{H}]\text{-Vb}$ accumulation in RBE4 cells. When cells were transfected with the wild-type form of SphK-1, the accumulation of

[³H]-Vb within the cells was significantly lower than in the mock-transfected cells (Fig. 4B). However, the overexpression of the dominant negative form of SphK-1 (G82D) did not modify the [³H]-Vb accumulation in RBE4 cells (Fig. 4B). These results indicate that the overexpression of SphK-1 induces the expression of functional P-gp.

Stimulation of P-gp transport activity by S1P in RBE4 cells: We also determined the impact of S1P on P-gp-mediated [³H]-Vb efflux. RBE4 cells were incubated with 1 μM S1P for 1 hour and [³H]-Vb accumulation was assayed. As shown on figure 5, the treatment with S1P stimulated [³H]-Vb efflux from cells. By Western blot analysis, we determined that P-gp expression was unaffected by the treatment of RBE4 cells with S1P (data not shown).

We next determined whether the overexpression of SphK-1 could also stimulate the P-gp transport activity through the formation of S1P in the RBE4 cell line. RBE4 cells were transfected with SphK-1-GFP plasmid or treated with 1 μM S1P, in presence or absence of 10 μM DMS, a specific inhibitor of SphK-1 (Fig. 5). The addition of DMS partially antagonized the effect of the overexpression of SphK-1 on the [³H]-Vb accumulation. The partial effect of DMS on transfected cells can be explained by the SphK-1-mediated upregulation of P-gp expression in RBE4 cells. However, S1P-mediated stimulation of P-gp function in RBE4 cells was unaffected by the addition of DMS (Fig. 5).

To further characterize the SphK-1-mediated regulation of P-gp, RBE4 cells were also pretreated for 16 hours with pertussis toxin (PTX) in order to block the function of S1P receptors at the cell surface. The PTX treatment partially antagonized the SphK-1-mediated stimulation of the [³H]-Vb efflux in RBE4 cells (Fig. 5). This suggests that SphK-1 modulates P-gp function through the upregulation of the protein but also through the S1P-mediated stimulation of P-gp transport activity. The PTX

treatment fully antagonized the S1P-mediated stimulation of P-gp function in RBE4 cells.

Stimulation of P-gp functions through S1P-1 and S1P-3 receptors in RBE4 cells:

Because PTX inhibited the S1P-mediated modulation of the P-gp transport activity, we evaluated which of the two Gi-associated S1P receptors (i.e. S1P-1 and S1P-3) could be implicated in the regulation of this phenomenon. Thus, to identify which one is responsible for the effect of S1P on P-gp transport activity, RBE4 cells were treated with antisens oligonucleotides raised against S1P-1 or S1P-3. As shown on figure 6, the scrambled oligonucleotides did not affect the S1P-mediated stimulation of the [³H]-Vb transport in RBE4 cells. However, both S1P-1 and S1P-3 antisens oligonucleotides partially antagonized the S1P-mediated stimulation of the P-gp function in RBE4 cells. These results indicate that the S1P-mediated stimulation of P-gp transport activity is transduced by both S1P-1 and S1P-3 receptors in RBE4 cells.

DISCUSSION

The ATP-dependent P-glycoprotein (P-gp) lies on the luminal membrane of the brain capillary endothelial cells that form the blood–brain barrier (BBB) and is a key element in the extrusion of structurally dissimilar compounds from the brain EC into the blood vessel lumen. P-gp also plays a crucial role in the acquisition and the maintenance of the MDR phenotype in brain EC (Regina et al. 2001). Here, we are reporting that SphK-1, which is also implicated in the development of brain tumors (Van Brocklyn et al. 2005), regulates P-gp expression and transport activity, through the generation of both intracellular and extracellular S1P, in brain EC.

Since the upregulation of P-gp expression is a common feature in the acquisition of the MDR phenotype (Henson et al. 1992), the increase in P-gp expression and activity induced by SphK-1 suggests that this kinase plays a crucial role in the setting of the MDR phenotype. This is in agreement with other studies that have also demonstrated the importance of the SphK-1 for the MDR phenotype. For instance, it has been showed that doxorubicin-sensitive HL-60 cells display SphK-1 inhibition coupled with ceramide generation. In contrast, chemoresistant HL-60 cells, that express P-gp, have sustained SphK-1 activity and do not produce ceramide (Bonhoure et al. 2005). Moreover, camptothecin resistant PC3 cells exhibit higher SphK-1 activity and also elevated expression of S1P receptors, S1P-1 and -3, as compared with those of sensitive cells (Akao et al. 2006). Altogether, these data and our own results confirm the major role of SphK-1 for the MDR phenotype acquisition. However, our data are the first results showing that SphK-1 could be involved in the regulation of P-gp at the BBB.

Interestingly, the overexpression of SphK-1 has been shown to be strongly associated to inflammation (Baumruker et al. 2005; Billich et al. 2005; Pyrko et al. 2005). The higher expression of SphK-1 in brain EC isolated from brain tumors

compared to normal brain EC suggests that brain tumor-derived EC undergo a chronic inflammation, as previously described (Deininger and Schluesener 1999). Cyclooxygenase-2 (COX-2) is one of the major inflammatory factors (Zha et al. 2004; Monnier et al. 2005). COX-2 has been shown to be upregulated by SphK-1 (Pettus et al. 2002) and to stimulate P-gp expression (Patel et al. 2002). As our results clearly show that the overexpression of SphK-1 leads to the upregulation of P-gp, this stimulation of P-gp expression in RBE4 could be dependent of COX-2. This concern is currently under investigation in our lab.

By RT-PCR, we demonstrate that the overexpression of SphK leads to the overexpression of the *mdr1-b* gene in RBE4 cells. The upregulation of *mdr1-b* has been associated to a cell differentiation phenotype. Thus, undifferentiated alveolar epithelial type II cells do not express *mdr1-b* gene, whereas differentiated alveolar epithelial type II cells do (Campbell et al. 2003). We already have described that the brain tumor endothelium displays a different molecular phenotype than the normal brain endothelium, including the upregulation of *mdr1-b* (Regina et al. 2003). Therefore, the brain-tumor mediated SphK overexpression in brain EC might lead to the tumoral differentiation of the cerebral endothelium characterized, unless in part, by the upregulation of *mdr1-b* gene.

It has been clearly demonstrated that the current use of chemotherapy to treat brain tumors is limited by the expression of P-gp in cancer cells (Ozben 2006). The expression of P-gp in EC from the BBB (Beaulieu et al. 1997) also limits the penetration of chemotherapeutic drugs in the brain, decreasing the efficacy of brain tumor treatments. In this paper, we have demonstrated that SphK-1 and its product S1P stimulate P-gp transport activity in EC from brain capillaries. This suggests that SphK-1 could affect the efficacy of current brain tumor treatments through the modulation of P-gp expression. The use of SphK-1 inhibitors could therefore favor

the penetration of drugs into the CNS, by inhibiting the SphK-1-mediated upregulation of P-gp. Moreover, they could also affect brain tumors themselves. Indeed, the overexpression of SphK-1 observed in brain tumor cells (Van Brocklyn et al. 2005) and in brain tumor-derived EC leads to the increase of S1P concentration in the brain. Because S1P stimulates both the invasiveness and the proliferation of brain tumor cells (Van Brocklyn et al. 2002; Van Brocklyn et al. 2003), SphK-1 inhibitors will block the development of brain tumors by decreasing the overall S1P concentration in the brain. The specific targeting SphK-1 in brain EC and brain tumors will reduce the development of brain tumors through the decrease of S1P and facilitate the penetration of chemotherapeutic drugs in the brain by inhibiting the upregulation of P-gp in brain EC. Consequently, SphK-1 inhibitors represent new antitumor weapons and will be of great help for the treatment of brain tumors.

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FIGURE LEGENDS

Figure 5.1: Upregulation of SphK-1 in brain EC isolated from brain tumors. (A) Endothelial cells were isolated from normal rat brain or glioma implanted orthotopically as described in the Material and Methods section, and cells lysates were subjected to Western blot analysis for SphK-1 expression. β -actin was detected as a loading control. (B) The expression of SphK-1 was analysed by densitometry using the IPLabGel software. Graph represents means quantifications $\pm$ SEM of at least three gels.

Figure 5.2: SphK-1-mediated increase of intracellular and extracellular S1P in RBE4 cells. RBE4 cells were transiently transfected with a cDNA plasmid encoding SphK-1-GFP or the pcDNA (3.1+) empty plasmid (mock). (A) Phase contrast (PC) and fluorescence views of mock or SphK-1-GFP transfected RBE4 cells. (B) Extracellular and intracellular S1P was measured in control and RBE4 cells as described in the Material and Methods section. ** indicate statistically significant differences ($P < 0.05$) compared to the control cells.

Figure 5.3: Upregulation of P-gp expression by the active form of SphK-1 in RBE4 cells. (A) RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP or pcDNA (3.1+) empty plasmid (mock). SphK-1 and P-gp were detected by Western blot analysis. (B) RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP, the dominant-negative form SphK-1-GFP (G82D) or the pcDNA (3.1+) empty plasmid (mock). Total RNA was extracted as described in the Materials and Methods section and *SphK-1*, *mdr-1a*, *mdr-1b* cDNA fragments were amplified by RT-PCR using specific oligonucleotides. β -tubulin was amplified as a loading control.

Figure 5.4: SphK-1-mediated decrease of [³H]-Vb accumulation in RBE4 cells.

(A) [³H]-Vb accumulation was measured in presence and absence of various P-gp inhibitors (CsA: 2.5 μ M; Verapamil: 10 μ M; PSC833: 5 μ M) or MRPs inhibitors (Probenecid: 10 μ M and Indomethacin: 10 μ M). (B) RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP, the dominant-negative form SphK-1-GFP (G82D) or the pcDNA (3.1+) empty plasmid (mock). [³H]-Vb accumulation was measured as described in the Method section. Results were expressed as the percentage of [³H]-Vb accumulation measured in absence of P-gp inhibitors. Graph represents means quantifications $\pm$ SEM of at least three independent experiments. ** indicate statistically significant differences ($P < 0.05$) compared to the control cells.

Figure 5.5: Modulation of P-gp function by SphK-1 and S1P in RBE4 cells.

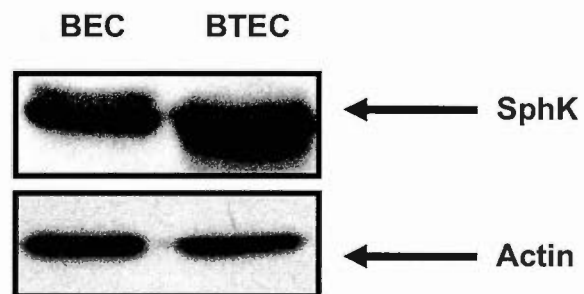
RBE4 cells were transiently transfected with a cDNA plasmid encoding the wild-type form SphK-1-GFP or pcDNA (3.1+) empty plasmid (mock) or treated with S1P 1 μ M for 1 hour and [³H]-Vb accumulation was measured. Cells were pretreated with either PTX at 20 ng/ml for 16 hours or DMS at 20 μ M for 1 hour. Graph represents means quantification $\pm$ SEM of at least three independent experiments. ** indicate statistically significant differences ($P < 0.05$) compared to the control cells.

Figure 5.6: S1P-mediated stimulation of P-gp transport activity in RBE4 cells through S1P-1 and S1P-3 receptors.

[³H]-Vb accumulation was measured in RBE4 cells transiently transfected with 200 nM of antisense (AS) oligonucleotides against S1P-1 and S1P-3, or with their scrambled controls (S), and used 72 hours post-transfection. (A) S1P-1 (S) and (AS) or (B) S1P-3 (S) and (AS) transfected cells were treated or not with S1P 1 μ M. Graph represents means quantification $\pm$ SEM of at least three independent experiments. ** indicate statistically significant differences ($P < 0.05$) compared to the control cells.

Figure 1: Upregulation of SphK-1 in brain EC isolated from brain tumors

A)



B)

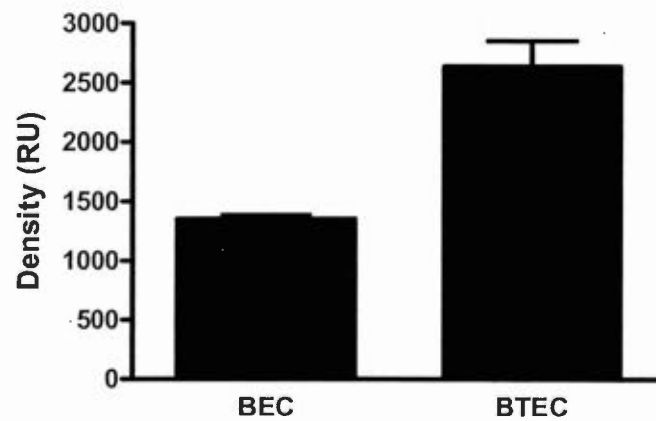
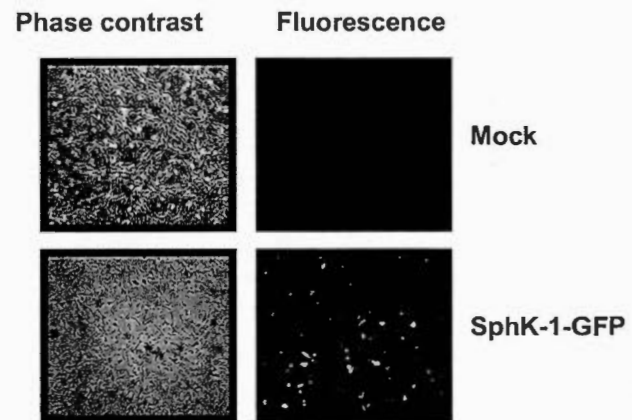


Figure 2: SphK-1-mediated increase of intracellular and extracellular S1P in RBE4 cells

A)



B)

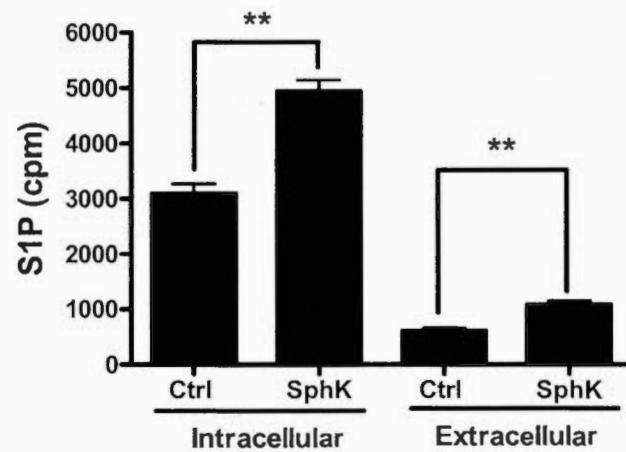
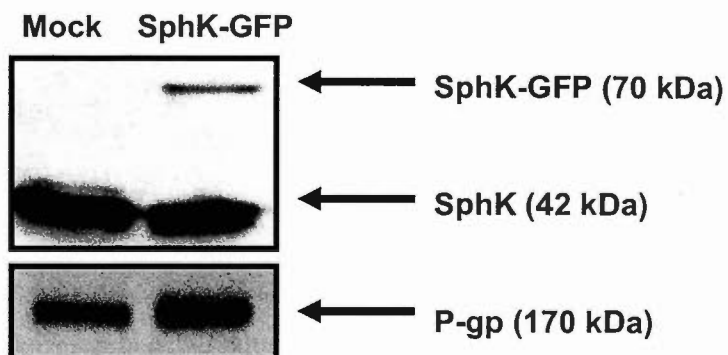


Figure 3: Upregulation of P-gp expression by the active form of SphK-1 in RBE4 cells

A)



B)

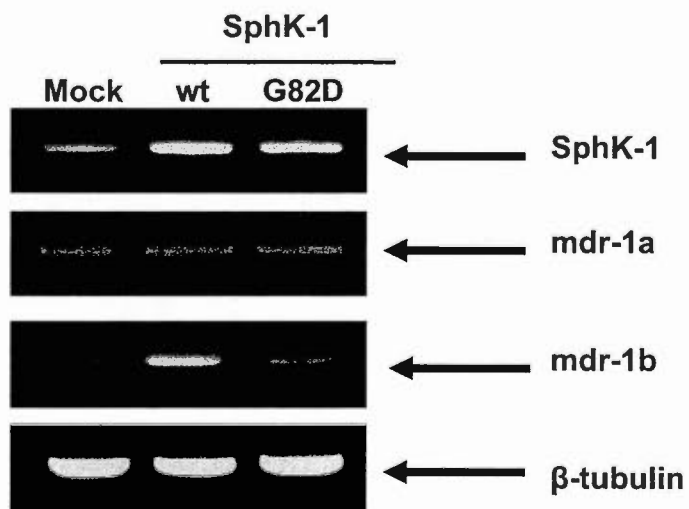
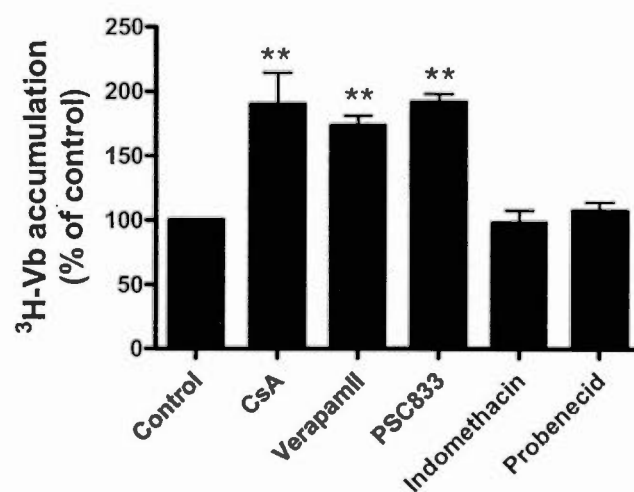


Figure 4: SphK-1-mediated decrease of [3 H]-Vb accumulation in RBE4 cells

A)



B)

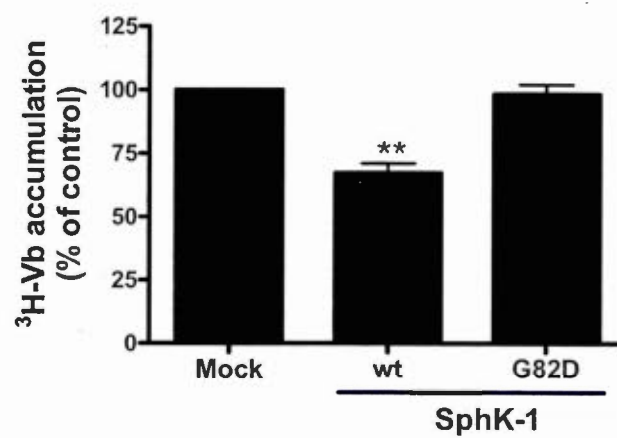


Figure 5: Modulation of P-gp function by SphK-1 and S1P in RBE4 cells

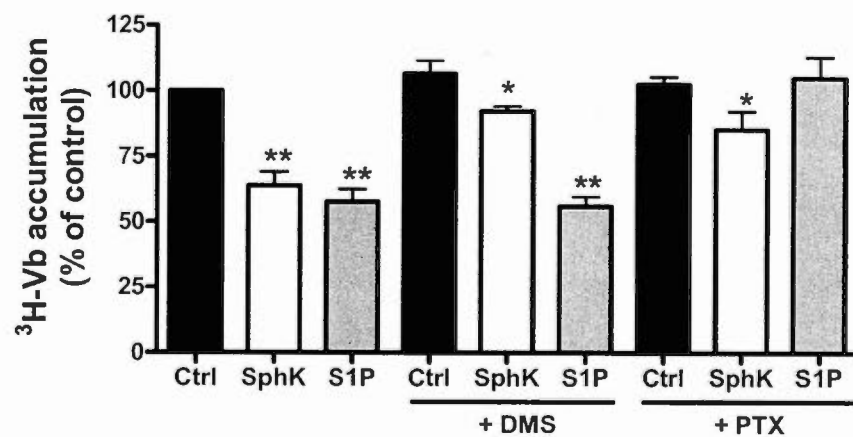
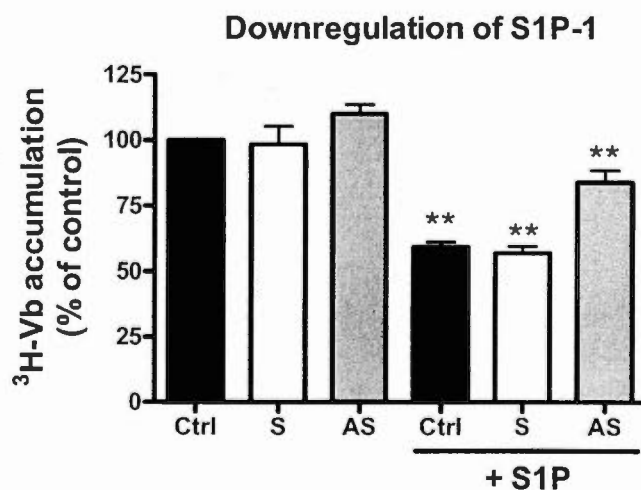
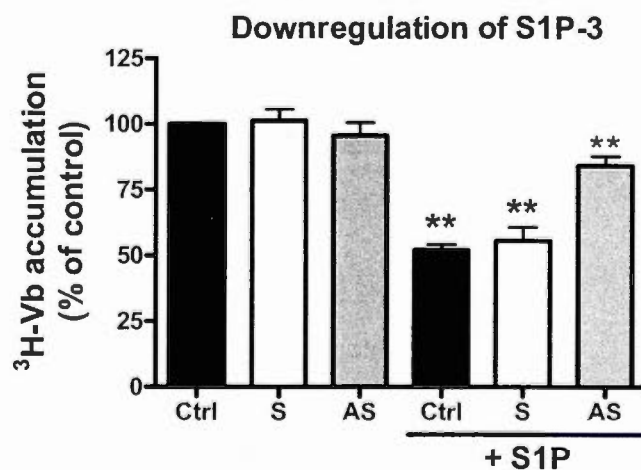


Figure 6: S1P-mediated stimulation of P-gp transport activity in RBE4 cells through S1P-1 and S1P-3 receptors

A)



B)



CHAPITRE III: DISCUSSION

Le cancer est la deuxième cause de mortalité dans le monde industrialisé après les accidents cardiovasculaires mais pourrait dans un futur proche occuper la première place de ce triste palmarès. C'est déjà le cas au Québec ! Cette maladie touche environ 10 millions de personnes dans le monde. La découverte de nouvelles thérapies pour cette maladie est, par conséquent, nécessaire afin de la combattre plus efficacement.

Molécules naturelles et traitements des tumeurs cérébrales.

Le thé vert représente l'un des aliments dont les propriétés chimiopréventives sont les mieux documentées. Les molécules les plus actives du thé vert sont les polyphénols ou catéchines (Demeule et al. 2002b). Dans notre première étude, nous nous sommes intéressés aux effets de l'EGCG, la principale catéchine du thé vert, sur les propriétés d'adhérence et de migration des cellules de médulloblastome DAOY. Il a déjà été démontré que l'EGCG inhibe l'adhérence et l'étalement des cellules de mélanome et de carcinome pulmonaires murins sur la fibronectine (Sazuka et al. 1998). Les auteurs ont notamment démontré que l'EGCG interagit avec le domaine C-terminal de la fibronectine, inhibant du même coup, de façon compétitive, l'interaction des cellules avec leurs sites de liaison sur la fibronectine (Sazuka et al. 1998). Le même type d'inhibition compétitive a aussi été démontré pour l'adhérence de cellules de mélanome sur la laminine (Suzuki and Isemura 2001). Cependant, les auteurs ne se sont pas intéressés à l'effet potentiel de l'EGCG sur les protéines d'adhérence cellulaire, telles que les intégrines.

Dans notre première étude, nous avons démontré que l'EGCG régule, tant au niveau transcriptionnel que traductionnel, l'expression de l'intégrine $\beta 1$ dans les cellules DAOY de médulloblastome. Cette étude est donc la première à démontrer

que l'EGCG peut affecter l'adhérence cellulaire autrement que par l'interaction avec les molécules de la MEC. Bien que les mécanismes précis de cette régulation ne soient pas connus, nous pouvons penser que l'augmentation de l'expression génique de l'intégrine $\beta 1$ est le résultat combiné des effets de l'EGCG sur diverses protéines impliquées dans la régulation de l'expression des gènes. En effet, il a été démontré que l'EGCG peut moduler la phosphorylation des MAPKinases (Chen et al. 2000; Chung et al. 2001), bloquer l'activation de NF κ B (Hou et al. 2004b) et interagir directement avec l'ADN et l'ARN afin de moduler l'expression des gènes dans la cellule (Kuzuhara et al. 2006). Par conséquent, d'autres études seront nécessaires afin de mettre à jour le mécanisme moléculaire précis par lequel l'EGCG stimule l'expression de l'intégrine $\beta 1$ dans les cellules DAOY.

Les médulloblastomes sont principalement traités par chirurgie et radiothérapie (Freeman et al. 2002). La chirurgie intracrânienne est une chirurgie très lourde et la radiothérapie détruit aussi les tissus sains autour de la tumeur puisque tout le névraxe est irradié de façon prophylactique (Jakacki 2005). L'utilisation de fortes doses de chimiothérapie est proscrite en raison d'effets secondaires importants, bien que son utilisation adjuvante ait démontré des effets positifs sur la survie des patients (Rutkauskiene and Labanauskas 2005). Il est donc nécessaire de trouver le moyen de réduire les doses de radiothérapie afin d'augmenter la fréquence des traitements. Dans cette optique, l'association des thérapies conventionnelles avec la nutrathérapie a permis de faire évoluer le traitement des médulloblastomes.

Les médulloblastomes sont des tumeurs radiosensibles (Leith et al. 1994; McLaughlin et al. 2006). Cependant, les doses efficaces nécessaires à leur destruction (25 à 30 Gys) sont encore trop importantes pour ne pas endommager les structures nerveuses du cervelet. Or, il a été démontré que l'EGCG augmente la radiosensibilité des tumeurs cérébrales (McLaughlin et al. 2006). On peut donc penser que l'utilisation simultanée de la nutrathérapie et de la radiothérapie permettrait de

radiosensibiliser les cellules tumorales et ainsi, de diminuer les doses de radiations nécessaires à la destruction des cellules cancéreuses. De plus, il a été démontré que l'irradiation stimule les propriétés angiogéniques des cellules endothéliales et favorise ainsi la récurrence tumorale (Hast et al. 2002; Mirsky et al. 2002). Or, l'EGCG antagonise efficacement la tubulogénèse des cellules endothéliales induite par l'irradiation (Annabi et al. 2003). Par conséquent, la mise en place d'une thérapie multimodale associant chirurgie, radiothérapie, chimiothérapie et nutrathérapie pourrait permettre d'augmenter sensiblement l'efficacité des traitements conventionnels des médulloblastomes et autres tumeurs cérébrales.

La croissance des tumeurs cérébrales requiert une angiogénèse soutenue afin d'assurer un apport en oxygène et en nutriments suffisant pour répondre aux besoins des cellules cancéreuses en division. Les thérapies antiangiogéniques sont donc un complément essentiel aux thérapies antitumorales. Le blocage des fonctions d'adhérence des intégrines vasculaires par des peptides RGD synthétiques est une des stratégies mises au point pour stopper la croissance des vaisseaux sanguins tumoraux (Strieth et al. 2006). L'efficacité de ces peptides synthétiques est très variable et leur spécificité est relativement faible, ce qui pose un problème important pour leur utilisation clinique. Or, on a découvert, il y a quelques années, que certains venins de serpent contiennent de nombreux peptides naturels capables de bloquer spécifiquement et, avec une efficacité 500 à 2000 fois supérieure à celle des peptides RGD synthétiques, la fonction des intégrines vasculaires, (Huang 1998; Lu et al. 2003).

Le blocage de la fonction des intégrines vasculaires par les peptides isolés de venins de serpents affecte en tout premier lieu le processus d'hémostase. En effet, ces peptides sont capables d'activer ou d'inhiber l'agrégation plaquettaire, mais peuvent aussi affecter directement l'endothélium vasculaire (Lu et al. 2005; Marsh and Williams 2005). Cette observation a permis d'utiliser ces peptides pour le traitement

des thromboses et autres pathologies vasculaires (Pal et al. 2002). Cependant, puisque parmi les cibles moléculaires de ces peptides se trouvent les intégrines vasculaires αV et $\alpha 5\beta 1$ (Huang et al. 2001; Wijeyewickrema et al. 2005), l'idée de les utiliser dans le cadre de thérapies antiangiogéniques du cancer a rapidement germé (Golubkov et al. 2003).

Dans notre deuxième étude, nous avons caractérisé les propriétés antiangiogéniques *in vivo* et *in vitro* de la lébectine, une lectine de type C (CLP) isolée du venin de la vipère *Macrovipera lebetina*. Nos résultats sont les seconds à mettre en évidence les propriétés antiangiogéniques d'une CLP. En effet, il a déjà été démontré que l'agkistine, une CLP isolée du venin de *Agkistrodon acutus*, inhibe l'angiogenèse *in vivo* (Yeh et al. 2000). De nombreux autres peptides, principalement des désintégrines, ont aussi démontré leur capacité à bloquer le processus angiogénique (Golubkov et al. 2003; Olfa et al. 2005). Cependant, dans le cas de ces peptides, les concentrations nécessaires à inhiber l'angiogenèse sont très supérieures aux concentrations efficaces de la lébectine. Par conséquent, la lébectine est le peptide isolé de venin de serpent dont l'efficacité antiangiogénique est la plus importante jamais rapportée à ce jour. Ces résultats viennent compléter une autre étude effectuée dans notre laboratoire concernant les propriétés antitumorales de la lébectine (Sarray et al. 2004). Par conséquent, l'ensemble de ces données ouvre la voie à la caractérisation des propriétés antiangiogéniques d'autres CLPs.

Nos résultats ont montré que de faibles concentrations de lébectine affectent spécifiquement les fonctions d'adhérence des intégrines $\alpha 5\beta 1$ et $\alpha V\beta 3$. Ces deux intégrines jouent un rôle crucial lors de l'angiogenèse tumorale. En effet, l'intégrine $\alpha 5\beta 1$ est fortement exprimée dans les vaisseaux sanguins des tumeurs alors qu'elle n'est pas exprimée dans les vaisseaux sanguins normaux (Kim et al. 2000b). L'intégrine $\alpha V\beta 3$, quant à elle, est fortement exprimée dans les vaisseaux sanguins des glioblastomes alors qu'on ne peut la détecter dans les vaisseaux sanguins non

tumoraux (Gingras et al. 1995; Gladson 1996). Par conséquent, l'utilisation clinique de la lébectine permettrait de cibler très spécifiquement les vaisseaux sanguins en formation dans les tumeurs sans affecter les vaisseaux sanguins non tumoraux.

Même si de nombreux peptides isolés de venins de serpent ont démontré leur efficacité antitumorale et antiangiogénique dans des expériences *in vitro* et/ou *in vivo* (Huang et al. 2001; Golubkov et al. 2003; Trochon-Joseph et al. 2004), peu d'entre eux ont prouvé leur efficacité dans des modèles animaux complexes de tumeurs implantées. A ce jour, les résultats les plus prometteurs concernent la contortrostatine, une désintégrine isolée du venin de *Agkistrodon contortrix contortrix*. Ce peptide inhibe très efficacement la croissance des gliomes (Pyrko et al. 2005), des carcinomes mammaires (Zhou et al. 2000), des tumeurs ovariennes (Markland et al. 2001) et des mélanomes (Trikha et al. 1994) implantés chez la souris. Il est important de noter que cette désintégrine est très bien acceptée par les animaux et ne montre pas d'effets adverses sur le système neurologique, ce qui suggère une faible antigénicité du peptide. L'activité antitumorale de la contortrostatine, quant à elle, semble être principalement liée à son pouvoir antiangiogénique, la contortrostatine liant l'intégrine $\alpha V\beta 3$ à la surface des cellules endothéliales (Golubkov et al. 2003).

Comme cela a été signalé ci-dessus, les traitements actuels de radiothérapie et de chimiothérapie des tumeurs cérébrales présentent des effets secondaires très importants et, dans le cas des tumeurs cérébrales pédiatriques, peuvent induire des séquelles neurologiques catastrophiques. De plus, la chimiothérapie se heurte à la présence de la barrière hématoencéphalique qui bloque le passage des molécules actives vers le cerveau (Regina et al. 2001). Par conséquent, les thérapies antiangiogéniques représentent un espoir important pour les patients atteints d'une tumeur cérébrale puisque ces thérapies ciblent directement l'endothélium vasculaire, ce qui permet d'ignorer la présence de la barrière hématoencéphalique. L'effet de la contortrostatine sur le développement *in vivo* des gliomes représente la toute première

étude validant l'efficacité des thérapies antiangiogéniques basées sur l'utilisation des venins de serpents pour le traitement des tumeurs cérébrales. L'efficacité thérapeutique des peptides isolés de venins de serpent, associée à leur bonne tolérance par l'organisme, suggère donc que ces molécules pourraient devenir des armes extrêmement efficaces afin de lutter contre les tumeurs cérébrales.

Sphingosine-1-phosphate et endothélium cérébral.

La sphingosine-1-phosphate (S1P) est un sphingolipide qui a été caractérisé pour son rôle au cours de l'hémostase (English et al. 2000). En effet, lors d'une blessure, l'activation plaquettaire induit la libération dans le sang de la S1P présente dans les plaquettes (Igarashi and Yatomi 1998). La S1P libérée stimule alors les cellules endothéliales vasculaires qui prolifèrent et migrent vers la source de S1P, autrement dit le caillot plaquettaire (Panetti et al. 2000). Cette activation des cellules endothéliales favorise le processus de cicatrisation.

Si le rôle proangiogénique de la S1P au niveau des cellules endothéliales périphériques est clairement établi (Panetti et al. 2000), son rôle au niveau de l'endothélium cérébral est encore peu documenté. Dans notre troisième étude, nous avons tenté de comprendre de quelle manière la S1P régule le processus angiogénique cérébral dans des conditions physiologiques. Nos résultats ont montré, de façon très surprenante, que la S1P inhibe les propriétés angiogéniques des cellules endothéliales cérébrales. Bien que la littérature scientifique regorge d'études démontrant le rôle proangiogénique de la S1P, il est important de remarquer que nos travaux ne vont pas à l'encontre des données déjà publiées mais permettent plutôt de les compléter. En effet, nos résultats montrent que la S1P inhibe la migration et la tubulogenèse des cellules endothéliales cérébrales, mais stimule les propriétés angiogéniques des cellules endothéliales non cérébrales dans des conditions physiologiques.

La microvascularisation cérébrale joue un rôle majeur dans la protection du système nerveux central (Takahashi and Macdonald 2004). Les cellules endothéliales cérébrales, les cellules gliales et les cellules nerveuses sont d'ailleurs organisées en unités neurovasculaires (Allan 2006). Par conséquent, toute modification dans le fonctionnement normal du tissu nerveux affectera le tissu endothélial et *vice versa*. Dans des conditions physiologiques, l'endothélium vasculaire cérébral est une structure extrêmement stable dont le rôle est de vasculariser et de protéger le cerveau grâce à la mise en place de la barrière hématoencéphalique (BHE). L'angiogenèse cérébrale n'est donc pas un phénomène fréquent au niveau cérébral et doit donc être perçue comme une réponse de l'endothélium à un état pathologique du cerveau. L'ischémie cérébrale ou le développement tumoral sont deux exemples de pathologies cérébrales entraînant une réponse angiogénique de la part de l'endothélium cérébral (Greenberg and Jin 2005). Dans les deux cas, les cellules nerveuses ou gliales se retrouvent dans des conditions d'hypoxie. En réponse au manque d'oxygène, les cellules sécrètent du VEGF qui va stimuler le processus angiogénique cérébral afin de réoxygéner le cerveau (Sun and Guo 2005). Le VEGF, s'il permet la réoxygénation du cerveau, affecte aussi l'intégrité de la BHE puisqu'il augmente fortement sa perméabilité (Kilic et al. 2006). Par conséquent, les cellules endothéliales cérébrales, dans des conditions physiologiques, semblent s'être adaptées afin de ne pas répondre à une stimulation angiogénique de la S1P lors des processus d'hémostase périphériques. L'effet antiangiogénique de la S1P au niveau de l'endothélium vasculaire cérébral permet de maintenir l'intégrité de la BHE et de protéger le cerveau d'une stimulation angiogénique qui lui serait délétère. Ceci suggère donc un rôle neuroprotecteur de la S1P dans des conditions physiologiques.

La perméabilité de l'endothélium vasculaire influe énormément sur ses propriétés de barrière. A l'inverse du VEGF, il a été démontré que la S1P diminue la perméabilité vasculaire *in vivo* et *in vitro* (McVerry and Garcia 2005; Singleton et al. 2005), augmentant de fait la fonction de barrière des endothéliums. De plus, nous

avons démontré que la S1P stimule l'activité de transport de la glycoprotéine P (P-gp) dans les cellules endothéliales cérébrales. La principale fonction de la P-gp au niveau de la BHE est d'extruder les xénobiotiques hors du compartiment cérébral, ce qui confère à cette protéine un rôle de protection directe du cerveau. La stimulation de son activité de transport par la S1P, associée à l'effet de la S1P sur la perméabilité vasculaire, renforce encore l'idée d'une implication de la S1P dans la protection du cerveau dans des conditions physiologiques.

Nos résultats ont montré que les cellules endothéliales cérébrales n'expriment pas MT1-MMP, une métalloprotéase de matrice de type membranaire, dans les conditions physiologiques. Or, il a été démontré que la S1P coopère avec MT1-MMP afin de stimuler les propriétés angiogéniques des cellules endothéliales (Langlois et al. 2004). Puisque MT1-MMP est fortement exprimée dans les cellules endothéliales non cérébrales au niveau desquelles la S1P exerce une activité proangiogénique, l'effet antiangiogénique de la S1P au niveau de l'endothélium cérébral peut s'expliquer par l'absence de MT1-MMP dans les cellules endothéliales cérébrales. De plus, nos résultats démontrent que la surexpression de MT1-MMP dans les cellules endothéliales cérébrales renverse l'effet antiangiogénique de la S1P renforçant encore le rôle majeur de MT1-MMP dans les fonctions proangiogéniques de la S1P.

Les tumeurs cérébrales, telles que les glioblastomes, sont parmi les tumeurs les plus vascularisées (Fischer et al. 2005). De plus, l'expression de la sphingosine kinase (SphK), l'enzyme responsable de la synthèse de la S1P, est fortement augmentée dans de nombreuses tumeurs, et plus particulièrement dans les tumeurs cérébrales telles que les glioblastomes (French et al. 2003; Van Brocklyn et al. 2005). En outre, il a été démontré que l'expression de la SphK dans les glioblastomes est corrélée à un faible pronostic pour les malades (Van Brocklyn et al. 2005). Par conséquent, il peut sembler paradoxal que l'expression de la SphK, et donc de la S1P, stimule le développement des tumeurs cérébrales alors que nos résultats démontrent

que la S1P inhibe fortement l'angiogenèse cérébrale. L'augmentation de la concentration de S1P dans le cerveau, en réponse à l'expression plus importante de la SphK, stimule en tout premier lieu la prolifération des cellules tumorales elles-mêmes (Van Brocklyn et al. 2002; Van Brocklyn et al. 2003). De plus, puisque les tumeurs cérébrales sont extrêmement vascularisées malgré la forte concentration de S1P dans le cerveau, il semble donc qu'en cas de tumeur, les cellules endothéliales cérébrales ne répondent plus à la S1P de la même façon que dans les conditions physiologiques. Nous avons montré qu'une des caractéristiques moléculaires des cellules endothéliales cérébrales normales est l'absence de MT1-MMP. Or, il a été démontré que cette protéine est fortement exprimée dans les cellules endothéliales cérébrales de glioblastomes (Munaut et al. 2003). Ceci suggère donc que les tumeurs modifient le phénotype des cellules endothéliales cérébrales, renversant ainsi l'effet antiangiogénique de la S1P. Cette modification du phénotype vasculaire cérébral, en réponse à l'environnement tumoral, a déjà été démontré dans notre laboratoire (Regina et al. 2003).

Dans des conditions physiologiques, l'endothélium cérébral assure la protection du cerveau. La structure étanche de la BHE permet de créer une véritable barrière physique entre le sang et le cerveau. A cela s'ajoute l'expression, au niveau des cellules endothéliales cérébrales, de transporteurs protéiques spécifiques tels que la glycoprotéine P (P-gp) qui renforcent la fonction de barrière de l'endothélium cérébral (Demeule et al. 2002a). Cependant, au cours du développement tumoral, l'endothélium cérébral perd peu à peu ses propriétés de barrière (Schneider et al. 2004) et permet la croissance de la tumeur grâce au processus d'angiogenèse cérébrale. De plus, si l'expression de la P-gp est un avantage certain dans des conditions physiologiques, elle représente un obstacle majeur pour le traitement des tumeurs cérébrales. En effet, de nombreuses molécules de chimiothérapie, substrats de la P-gp, ne passent pas la BHE car elles sont extrudées hors du cerveau par la P-gp (Regina et al. 2001).

Dans notre dernière étude, nous avons étudié la régulation de l'expression et de l'activité de transport de la P-gp par la SphK et la S1P au niveau des cellules endothéliales cérébrales. Nos résultats montrent que l'expression de la SphK est augmentée dans les cellules endothéliales isolées de tumeurs cérébrales. Ceci démontre encore une fois la spécificité phénotypique des cellules endothéliales au sein du lit vasculaire tumoral. De plus, la surexpression de la SphK dans les cellules endothéliales cérébrales entraîne l'augmentation de l'expression et de l'activité de transport de la P-gp. Puisque l'expression et l'activité de transport de la P-gp semblent, en partie au moins, être régulées par la SphK, l'utilisation simultanée d'inhibiteurs de la SphK et d'inhibiteurs de la P-gp pourrait permettre d'améliorer la pénétration des drogues de chimiothérapie dans le cerveau afin de traiter les tumeurs cérébrales. De plus, la surexpression de la SphK dans les cellules endothéliales cérébrales devrait aussi influencer sur le processus angiogénique cérébral. Par conséquent, la SphK représente une cible importante des thérapies antiangiogéniques. L'utilisation d'inhibiteurs de la SphK pour le traitement de certains cancers a déjà donné des résultats prometteurs, tant *in vitro* qu'*in vivo* (French et al. 2006). Malgré tout, l'efficacité de ces inhibiteurs pour l'amélioration des traitements contre les tumeurs cérébrales reste encore à être prouvée cliniquement.

L'identification de nouvelles molécules ciblant l'endothélium cérébral, associée à une meilleure connaissance de la régulation de ce tissu permet de mettre en place de nouvelles stratégies thérapeutiques afin de lutter contre les tumeurs cérébrales. Nous avons mis en évidence l'importance de la SphK au cours de développement des tumeurs cérébrales et son rôle potentiel au cours du processus angiogénique cérébral. De plus, nous avons démontré que la lébectine pouvait bloquer spécifiquement l'angiogenèse cérébrale. Par conséquent, l'association de la lébectine et des inhibiteurs de la SphK pourrait permettre d'augmenter

significativement l'efficacité des thérapies antiangiogéniques pour le traitement des tumeurs cérébrales.

Chapitre IV : Conclusions et perspectives.

Conclusions générales

Le traitement des tumeurs cérébrales représente un défi immense pour la médecine. La recherche de nouveaux traitements se révèle de plus en plus nécessaire. Au cours de notre étude, nous avons mis en évidence les propriétés antitumorales de certaines molécules naturelles présentes dans notre alimentation ou dans certains venins de serpents. Ces molécules présentent un grand intérêt clinique, que ce soit pour leur facilité d'utilisation ou pour leur spécificité d'action. Les molécules naturelles issues de notre alimentation ne présentent pas d'effets secondaires, comparées aux protocoles classiques de radiothérapie et de chimiothérapie, ce qui leur confère un avantage certain sur les traitements conventionnels. De plus, l'approche métronomique de la nutrathérapie permet de maintenir un niveau constant de molécules anticancéreuses dans l'organisme, ce qui n'est pas le cas pour les thérapies antitumorales classiques. Enfin, la mise en place d'une diète anticancéreuse permet d'augmenter la quantité de molécules actives dans l'organisme sans augmenter la prise de médicaments, ce qui représente un plus grand confort pour les patients. Quant aux peptides isolés de venins de serpents, ils se démarquent par leur activité spécifique au niveau de l'endothélium vasculaire et par leur très grande efficacité. Par conséquent, leur utilisation au cours des thérapies antiangiogéniques permet de cibler très précisément les vaisseaux sanguins en formation au cœur des tumeurs, sans affecter les vaisseaux sanguins matures. De plus, de très faibles concentrations de peptides suffisent généralement à bloquer totalement le processus angiogénique et le développement tumoral (Pyrko et al. 2005). Ceci permet donc de diminuer les risques liés aux propriétés antigéniques de ces peptides. Les molécules naturelles pourraient donc être extrêmement efficaces dans les traitements des tumeurs cérébrales, en ciblant tout à la fois les cellules tumorales et l'endothélium cérébral.

La mise en place de thérapies antiangiogéniques pour lutter contre les tumeurs cérébrales passe par une meilleure connaissance du fonctionnement de l'endothélium cérébral. Nous avons mis en évidence le rôle ambivalent de la S1P dans la régulation des propriétés angiogéniques et des propriétés de barrière de l'endothélium cérébral. Ainsi, nous avons démontré que, dans des conditions physiologiques, la S1P joue un rôle crucial dans la protection du cerveau. En plus de bloquer les propriétés angiogéniques des cellules endothéliales cérébrales, elle augmente les propriétés de barrière de l'endothélium cérébral, elle entraîne une vasoconstriction des vaisseaux cérébraux (Tosaka et al. 2001) et stimule l'activité de transport de la P-gp. Cependant, lors du développement d'une tumeur cérébrale, la S1P stimule la croissance des cellules cancéreuses et l'angiogenèse cérébrale. Ces données ont permis de mettre en évidence la spécificité phénotypique de l'endothélium cérébral normal et de l'endothélium cérébral tumoral, qui peut permettre d'expliquer les réponses différentes des cellules endothéliales normales et tumorales suite à une stimulation par la S1P. L'implication de la S1P et de la sphingosine kinase (SphK) au cours du développement des tumeurs cérébrales fait de ces deux molécules de nouvelles cibles potentielles pour les traitements anticancéreux.

Perspectives.

Nous avons décrit les propriétés antitumorales de l'EGCG dans le cas des médulloblastomes. Même si l'EGCG traverse la barrière hématoencéphalique (BHE) (Nakagawa and Miyazawa 1997; Suganuma et al. 1998), les concentrations intracérébrales sont relativement faibles. Or, les cellules endothéliales cérébrales sont directement en contact avec le sang et peuvent interagir facilement avec l'EGCG. Par conséquent, il serait intéressant d'évaluer l'effet de l'EGCG sur les propriétés angiogéniques des cellules endothéliales cérébrales. L'EGCG inhibe l'angiogenèse périphérique très efficacement (Fassina et al. 2004; Rodriguez et al. 2006). Par

conséquent, il serait important de savoir si l'EGCG exerce aussi un effet antiangiogénique sur l'endothélium cérébral.

Les peptides isolés de venins de serpents présentent un grand intérêt pour le traitement du cancer. Dans notre laboratoire, nous avons démontré les propriétés antitumorales et antiangiogéniques de la lébectine. Cependant, il reste encore à démontrer les effets de ce peptide sur le développement de tumeurs cérébrales chez l'animal. Dans un premier temps, il serait intéressant d'étudier le passage de la lébectine à travers la barrière hématoencéphalique (BHE). Après l'iodation de la lébectine, il nous serait possible de déterminer aisément le coefficient de passage de la lébectine à travers la BHE. Ceci permettrait de savoir si ce peptide pourrait affecter directement le développement des tumeurs cérébrales. Cependant, on peut aussi concevoir l'utilisation de ces peptides pour le traitement de toutes les maladies possédant une composante angiogénique. C'est le cas, par exemple, de la maladie d'Alzheimer. En effet, le peptide d'amyloïde β , caractéristique de cette maladie, stimule fortement l'angiogenèse cérébrale (Zand et al. 2005; Zlokovic 2005). Or, les intégrines αV participent activement à ce processus angiogénique (Grammas et al. 2006) et concourent donc au développement de la maladie. Par conséquent, l'utilisation de la lébectine pourrait permettre de freiner cette angiogenèse associée au développement de la maladie d'Alzheimer.

Nous avons déterminé que l'effet antiangiogénique de la SIP au niveau de l'endothélium cérébral était dû à l'absence de MT1-MMP dans les cellules endothéliales cérébrales. Or, MT1-MMP est exprimée dans les cellules endothéliales de glioblastomes (Munaut et al. 2003). Par conséquent, il serait intéressant de déterminer quels sont les facteurs tumoraux qui stimulent la transcription de MT1-MMP au niveau de l'endothélium cérébral. Il se pourrait aussi que le niveau d'oxygénation plus faible des cellules endothéliales au sein des glioblastomes stimule l'expression de MT1-MMP. Il a déjà été montré que l'expression de MT1-MMP

pouvait être régulée par l'hypoxie (Noda et al. 2005). En effet, l'expression de MT1-MMP est régulée par le facteur de transcription Egr-1 (Haas et al. 1999) et ce facteur est surexprimé en réponse à l'hypoxie tissulaire (Heiniger et al. 2003).

Nos résultats démontrent que l'activité de transport de la P-gp est augmentée en réponse à la S1P. Il serait particulièrement intéressant de comprendre par quelles voies de signalisation se fait l'activation de la P-gp. Le degré de phosphorylation de la P-gp pourrait aussi faire partie des régulations possibles de son activité suite à une stimulation par la S1P. En utilisant de l'ATP marqué au ^{32}P , nous pourrions aisément répondre à cette question. De plus, nos résultats ont montré que la surexpression de la sphingosine kinase stimule l'expression de la P-gp dans les cellules endothéliales cérébrales. Le mécanisme précis de cette régulation n'est pas connu. Il serait donc important de comprendre comment la surexpression de la sphingosine kinase régule l'expression du gène codant pour la P-gp, et de quelle manière la stimulation de l'expression de la P-gp dans les cellules endothéliales tumorales peut influencer sur le fonctionnement normal de la barrière hématoencéphalique. Il faudrait notamment déterminer si l'augmentation de l'expression génique de la P-gp dans les cellules RBE4 est due à la S1P intracellulaire, produite par la SphK, ou à la stimulation des récepteurs EDG par la S1P au niveau de la membrane plasmique.

Les travaux de cette thèse ont permis la caractérisation de nouvelles molécules naturelles aux propriétés antiangiogéniques et antitumorales. Leur utilisation clinique pourrait permettre de grandement améliorer les traitements actuels contre les tumeurs cérébrales grâce à la mise en place de thérapies multimodales, associant l'utilisation de molécules naturelles et les thérapies conventionnelles telles que la chimiothérapie ou la radiothérapie. De plus, nos travaux ont permis de mieux comprendre le rôle crucial de la S1P dans le fonctionnement normal des cellules endothéliales cérébrales et de mieux appréhender l'importance de l'endothélium vasculaire pour le

fonctionnement normal du cerveau mais aussi pour le développement des tumeurs cérébrales.

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